STUDIES OF THE RESPONSE OF MUSCLE INVASIVE BLADDER CANCER TO RADIOTHERAPY

by

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ABSTRACT

The purpose of this study was to investigate several biological markers that may predict the response of muscle invasive transitional cell carcinoma TCC of the bladder to radical radiotherapy. The specific markers chosen were tumour angiogenesis (CD31&CD34), tumour cell proliferation (Ki-67) and apoptosis (bcl-2), intratumour macrophage infiltration (CD68) and p53.

Archival formalin fixed paraffin embedded pre treatment bladder biopsies from 101 patients with muscle invasive TCC were obtained. All patients subsequently received radical radiotherapy as their only treatment for the disease. 4μm sections were graded according to the WHO grading system and staged by the TNM classification. Angiogenesis (CD31&CD34) counts were obtained using a 25-point Chalkley eyepiece graticule. Bcl-2 scored as positive and negative, while p53, Ki-67 and CD68 were estimated using an eyepiece graticule. The medical records were examined to assess the response of the tumour at the 3-month post radiotherapy cystoscopy and the long-term outcome. Patients were classified into two groups in two sections: the first section includes (1) those free of disease (no tumour detected in the bladder at the 3-month cystoscopy), (2) those with resistant disease (tumour present at 3 months). The second section includes (1) those with persistent or recurrent cancer in the bladder (tumour recurred after an initial 3 months negative check cystoscopy together with patients with resistant disease at 3 months), (2) those free of disease at all subsequent cystoscopies.

Detailed statistical analysis revealed that there were no association between any of the markers examined and the response to radiotherapy. MVD using CD34 was lower in higher stage tumours ($p=0.050$). Females, whilst representing only a small fraction (16) of the total of patients studied showed an inferior response to radiotherapy when compared to that of male patients ($p=0.048$). Higher median haemoglobin levels for the response group ($p=0.031$) was observed as well as a positive significant correlation between p53 (LI) expression and MIB-1 (LI) ($r=0.332$, $p=0.001$).
The Kaplan-Meier survival analysis shows that the survival time is significantly better for those who were exposed longer to radiotherapy (> 33 days) (Log Rank, $p=0.0246$). There was a significantly higher survival time for patients who have CD68 higher than 42.4 (log rank, $p=0.036$).

The study concluded that none of the selected markers could be used as prognostic value in determining patients most suitable for radiotherapy as primary treatment with curative intent for their bladder cancer. The finding of a poorer response in females is worthy of further study since, hormonal and anatomical influences may be important.

TABLE OF CONTENTS

Page

COPYRIGHT STATEMENT I

TITLE PAGE II

ABSTRACT III

TABLE OF CONTENTS IV

LIST OF TABLE VIII

LIST OF FIGURES XII

ACKNOWLEDGEMENTS XV

AUTHOR'S DECLERATION XVI

DEDICATION XVII

ABBREVIATIONS XVIII

CHAPTER ONE: INTRODUCTION

1.1: General consideration 1

1.1.1: The aetiology of bladder cancer 3

1.1.2: The epidemiology of bladder cancer 7

1.1.3: The histopathology of bladder cancer 9

1.1.4: Tumour grade 16

1.1.5: Tumour stage 19

1.1.6: Diagnostic procedure 25

1.1.6.1: Urine cytology and other diagnostic tests 28

1.1.6.2: Bladder cancer screening 34

1.1.7: The treatment of bladder cancer 37

1.1.7.1: Superficial disease (Ta;T1; CIS) 37

1.1.7.2: Muscle invasive disease (T2; T3; T4) 38

1.1.8: Chemotherapy 45

1.1.8.1: Neoadjuvant chemotherapy 45

1.1.8.2: Adjuvant chemotherapy 48

1.2: Angiogenesis 51
1.2.1: Physiological angiogenesis 53
1.2.2: Pathological angiogenesis 53
1.2.3: Angiogenic and anti-angiogenic factors 55
1.2.4: Methods for studying angiogenesis 60
1.2.5: Tumour angiogenesis 62
1.2.6: Angiogenesis and metastasis 68
1.2.7: Angiogenesis in bladder cancer 70

1.3: Radiotherapy 76
1.3.1: Radiotherapy in bladder cancer 79
1.3.2: Morbidity of radical radiotherapy 84

1.4: Prognostic markers 85
1.4.1: Monoclonal mouse anti-human endothelial cell, CD31&CD34 86
1.4.2: Monoclonal antibody Ki-67 antigen (MIB-1) 87
1.4.3: Monoclonal mouse anti-human p53 protein 88
1.4.4: Monoclonal mouse anti-human BCL-2 oncoprotein 91
1.4.5: Monoclonal mouse anti-human macrophage, CD68 93

1.5: Aims of the study 99
2.5.1: General objectives 99
2.5.2: Specific objectives 99

CHAPTER TWO: MATERIAL & METHODS

2.1: Patients 100
2.2: Immunohistochemistry 103
2.2.1: Reagents 103
2.2.2: Immunohistochemistry procedure 103
2.2.3: Vessel density determinations 108

2.2.4: Immunohistochemical analysis of bcl-2, p53, MIB-1 and CD68..... 112

2.3: Counting technique..... 117

2.4: Statistical analysis..... 119

CHAPTER THREE: RESULTS 120

3.1. SECTION ONE..... 121

3.1.1: The effects individually of age, sex, haemoglobin level and duration of treatment on the response to radiotherapy..... 122

3.1.2: The effects individually of tumour stage, tumour grade and site in the bladder on the response to EBRT..... 125

3.1.3: Tumour angiogenesis, Intratumour macrophage infiltration, p53, MIB-1, bcl-2 and the response to EBRT..... 127

3.1.4: Association between MVD, haemoglobin, macrophage infiltration, MIB-1, bcl-2, p53 and tumour characteristics..... 130

3.1.5: Association between other variables..... 140

3.1.6: Multivariate analysis of the effects of the variables with respect to the response to radiotherapy..... 155

3.2: SECTION TWO..... 156

3.2.1: The effects individually of age, sex, haemoglobin level and duration of treatment on the response to radiotherapy..... 157

3.2.2: The effect of tumour stage, tumour grade and site in the bladder on the response to EBRT..... 160

3.2.3: Tumour angiogenesis, Intratumour macrophage infiltration,p53, MIB-1, bcl-2 and the response to EBRT..... 162

3.2.4: Multivariate analysis of the effects of the variables with respect to the response to radiotherapy..... 165

4: Survival..... 166

CHAPTER FOUR: GENERAL DISCUSSION & CONCLUSION

4.1: General discussion..... 174

4.2: Conclusion..... 191

APPENDIX..... 192

LITERATURE CITED..... 212

LIST OF TABLE

Page

CHAPTER ONE:

Tab. 1. World Health Organization: Histological Classification of Urinary Bladder Tumours..... 14

Tab. 2. TNM Classification of Urinary Bladder Cancer..... 20

Tab. 3. Treatment Options for Muscle-Infiltrating Disease..... 39

Tab. 4. Randomised Trials of Preoperative Irradiation and Planned Cystectomy Vs. Radical Radiotherapy Alone in Muscle- Invading Bladder Cancers... 41

Tab. 5. Combination regimens of new agents in metastatic TCC..... 44

Tab. 6. Randomised phase III trials of neo-adjuvant chemotherapy..... 47

Tab. 7. Trials of adjuvant chemotherapy following cystectomy..... 49

Tab. 8. Physiological and pathological angiogenesis..... 54

Tab. 9. Examples of proangiogenic factors 56

Tab.10. Examples of antiangiogenic substances..... 57

Tab. 11. Models of angiogenesis..... 61

Tab.12. Pro- and anti-angiogenic factors described in bladder cancer..... 71

Tab.13. Studies of microvessel density (MVD) and prognosis in bladder cancer... 73

Tab.14. Five-year local control and overall survival in selected larger series after definitive radiotherapy for bladder cancer..... 81

CHAPTER THREE:

Tab. 3.1. Mean and median age by gender..... 120

Tab. 3.1.1. Patient's response to EBRT and crude survival during the follow up period..... 121

Tab. 3.1.2. Mean age (SD) by response to radiotherapy..... 122

Tab. 3.1.3. Gender of patients by response to EBRT..... 123

Tab. 3.1.4. Patients haemoglobin level by response to radiotherapy..... 123

Tab. 3.1.5. Number of days by response to radiotherapy..... 124

Tab. 3.1.6. Stage of tumour by response to EBRT..... 125

Tab. 3.1.7. Grade of tumour by response to EBRT..... 125

Tab. 3.1.8. Site of tumour by response to EBRT..... 126

Tab. 3.1.9. Median (IQR) of tumour angiogenesis by response to radiotherapy..... 127

Tab. 3.1.10. Mean (SD) of CD68 (LI) infiltrations by response to radiotherapy.... 128

Tab. 3.1.11. Median (IQR) of p53 (LI) infiltrations by response to radiotherapy.... 128

Tab. 3.1.12. Mean (SD) of MIB-1 (LI) by response to radiotherapy..... 129

Tab. 3.1.13. Response to radiotherapy by bcl-2..... 129

Tab. 3.1.14. Median (IQR) of MVD by stage of bladder TCC..... 130

Tab. 3.1.15. Median (IQR) of MVD by grade of bladder TCC..... 131

Tab. 3.1.16. Median (IQR) of MVD by site of bladder TCC..... 131

Tab. 3.1.17. Haemoglobin and stage of TCC..... 132

Tab. 3.1.18. Haemoglobin and grade of TCC..... 132

Tab. 3.1.19. Haemoglobin and site of TCC..... 133

Tab. 3.1.20. CD68 and stage of TCC..... 133

Tab. 3.1.21. CD68 and grade of TCC..... 134

Tab. 3.1.22. CD68 and site of TCC..... 134

Tab. 3.1.23. Association between bcl-2 and stage of bladder TCC..... 135

Tab. 3.1.24. Association between bcl-2 and grade of bladder TCC..... 135

Tab. 3.1.25. Association between bcl-2 and site of bladder TCC..... 136

Tab. 3.1.26. Association between MIB-1 (L I) and stage of bladder TCC..... 136

Tab. 3.1.27. Association between MIB-1 and grade of bladder TCC..... 137

Tab. 3.1.28. Association between MIB-1 and site of bladder TCC..... 137

Tab. 3.1.29. Association between p53 (LI) and stage of bladder TCC..... 138

Tab. 3.1.30. Association between p53 (LI) and grade of bladder TCC..... 138

Tab. 3.1.31. Association between p53 (LI) and site of bladder TCC..... 139

Tab. 3.1.32. Correlation between p53 (LI) and MVD in bladder TCC..... 140

Tab. 3.1.33. Correlation between MIB-1 (LI) and MVD in bladder TCC..... 141

Tab. 3.1.34. Correlation between p53 (LI) and MIB-1 in bladder TCC..... 142

Tab. 3.1.35. Correlation between p53 (LI) and CD68 (LI) in bladder TCC..... 143

Tab. 3.1.36. Correlation between CD31 and CD34 in bladder TCC..... 144

Tab. 3.1.37. Correlation between CD68 (LI) and CD31 in bladder TCC..... 145

Tab. 3.1.38. Correlation between CD68 (LI) and CD34 in bladder TCC..... 146

Tab. 3.1.39. Correlation between MIB-1 and CD68 (LI) in bladder TCC..... 147

Tab. 3.1.40. Correlation between haemoglobin level and MVD in bladder TCC.... 148

Tab. 3.1.41. Correlation between haemoglobin and CD68 (LI) in bladder TCC.... 149

Tab. 3.1.42. Correlation between haemoglobin and p53 (LI) in bladder TCC..... 150

Tab. 3.1.43. Correlation between haemoglobin and MIB-1 (LI) in bladder TCC... 151

Tab. 3.1.44. Correlation between Bcl-2 and MVD (CD31) in bladder TCC..... 152

Tab. 3.1.45. Correlation between Bcl-2 and MVD (CD34) in bladder TCC..... 152

Tab. 3.1.46. Correlation between Bcl-2 and CD68 in bladder TCC..... 153

Tab. 3.1.47. Correlation between Bcl-2 and p53 in bladder TCC..... 153

Tab. 3.1.48. Correlation between Bcl-2 and MIB-1 in bladder TCC..... 154

Tab. 3.1.49. Correlation between Bcl-2 and haemoglobin level in bladder TCC.... 154

Tab. 3.1.50. Predictors for response to radiotherapy..... 155

Tab. 3.2.1. Patient's response to EBRT..... 156

Tab. 3.2.2. Mean age (SD) by response to radiotherapy..... 157

Tab. 3.2.3. Gender of patients by response to EBRT..... 158

Tab. 3.2.4. Patients haemoglobin level by response to radiotherapy..... 158

Tab. 3.2.5. Number of days by response to radiotherapy..... 159

Tab. 3.2.6. Stage of tumour by response to EBRT..... 160

Tab. 3.2.7. Grade of tumour by response to EBRT..... 160

Tab. 3.2.8. Site of tumour by response to EBRT..... 161

Tab. 3.2.9. Median (IQR) of tumour angiogenesis by response to radiotherapy..... 162

Tab. 3.2.10. Mean (SD) of CD 68 (LI) infiltrations by response to radiotherapy.... 163

Tab. 3.2.11. Median (IQR) of p53 (LI) infiltrations by response to radiotherapy.... 163

Tab. 3.2.12. Mean (SD) of MIB-1 (LI) by response to radiotherapy..... 164

Tab. 3.2.13. Response to radiotherapy by bcl-2..... 164

Tab. 3.2.14. Predictors for response to EBRT..... 165

Tab. 4.1. Cut-off points of the variables..... 167

LIST OF FIGURES

CHAPTER ONE:

Fig. 1. Histology of the normal bladder..... 10

Fig. 2. Transitional epithelium..... 10

Fig. 3. Von Brunn's nests..... 12

Fig. 4. Cystitis cystica..... 12

Fig. 5. Papilloma of the urinary bladder 17

Fig. 6. Transitional cell carcinoma, grade I..... 17

Fig. 7. Transitional cell carcinoma, grade II..... 18

Fig. 8. Transitional cell carcinoma, grade III..... 18

Fig. 9. The relationship between the location and depth of the tumour and the stage..... 22

Fig. 10. Carcinoma in situ..... 24

Fig. 11. Surgeon's view of tumour (direct visualization of the tumour)..... 27

Fig. 12. Process of angiogenesis..... 52

Fig. 13. Angiogenesis balance..... 54

Fig. 14. The angiogenesis-signalling cascade..... 65

Fig. 15. Source of angiogenesis factors..... 67

CHAPTER TWO:

Fig. 2.1. Muscle invasive TCC..... 101

Fig. 2.2. Staining endothelium cells for CD31..... 110

Fig. 2.3. Staining endothelium cells for CD34..... 111

Fig. 2.4. Bcl-2 positive staining..... 113

Fig. 2.5. p53 positive staining..... 114

Fig. 2.6. MIB-1 positive staining..... 115

Fig. 2.7. CD68 positive cells..... 116

Fig. 2.8. Counting cells within stained sections..... 118

CHAPTER THREE:

Fig. 3.1. The distribution of studied bladder cancer patients by age at diagnosis.... 120

3.1 Section one

Fig. 3.1.1. Distribution of Age by response to radiotherapy..... 122
Fig. 3.1.2. Scatter plot of p53 Vs MVD (CD34)..... 140
Fig. 3.1.3. Scatter plot of MIB-1 Vs MVD (CD31)..... 141
Fig. 3.1.4. Scatter plot of p53 Vs MIB-1..... 142
Fig. 3.1.5. Scatter plot of p53 Vs CD68..... 143
Fig. 3.1.6. Scatter plot of CD31 Vs CD34..... 144
Fig. 3.1.7. Scatter plot of CD68 Vs MVD (CD31)..... 145
Fig. 3.1.8. Scatter plot of CD68 Vs MVD (CD34)..... 146
Fig. 3.1.9. Scatter plot of CD68 Vs MIB-1..... 147
Fig. 3.1.10. Scatter plot of haemoglobin level Vs MVD (CD34)..... 148
Fig. 3.1.11. Scatter plot of haemoglobin level Vs CD68..... 149
Fig. 3.1.12. Scatter plot of haemoglobin level Vs p53..... 150
Fig. 3.1.13. Scatter plot of haemoglobin level Vs MIB-1..... 151

3.2 Section two

Fig. 3.2.1. Age distribution by response to EBRT..... 157
Fig. 4.1. Mean (SD) for length of treatment..... 168
Fig. 4.2. Survival by length of treatment..... 168
Fig. 4.3. Survival times for CD68 (macrophage)..... 169
Fig. 4.4. Survival times for stage of tumour..... 169
Fig. 4.5. Survival times for grade of tumour..... 170
Fig. 4.6. Survival times for Haemoglobin level..... 170
Fig. 4.7. Survival times for CD31..... 171
Fig. 4.8. Survival times for CD34..... 171
Fig. 4.9. Survival times for gender..... 172
Fig. 4.10. Survival time by tumour proliferation (MIB-1)..... 172
Fig. 4.11. Survival time by bcl-2..... 173

Fig. 4.12. Survival time by p53 173

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AUTHOR'S DECLERATION

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Signed.....

Date...14..10..2002

DEDICATION

I would like to dedicate this work

To

my parents and my wife

ABBREVIATIONS

MVD:	Microvessels Density
TCC:	Transitional cell cancer
EBRT:	External beam radiotherapy
AIDS:	Acquired immunodeficiency syndrome
Rb1:	Retinoblastoma protein
HPV:	Human papilloma virus
TUR:	Transurethral resection
BCG:	Bacille Calmette-Guerin
WHO:	World Health Organization
TURBT:	Transurethral resection of the bladder tumour
TNM:	Tumour-node-metastases
CIS:	Carcinoma in situ
IVU:	Intravenous urography
CT:	Computerised tomography
MRI:	Magnetic resonance imaging
BTA:	Bladder-tumour-associated antigen test
FDP:	Fibrin/fibrinogen degradation products test
NMP22:	Nuclear matrix protein
FCM:	Flow cytometry
PPV:	Positive predictive values
EORTC-GU:	European Organisation for Research and Treatment of Cancer Genito-Urinary.
M-VAC:	(Methotrexate, vinblastine, doxorubicin and cisplatin)
BM:	Basement membrane
MVEC:	Microvascular endothelial cell
ECs:	Endothelial cells
VEGF:	Vascular endothelial growth factor
NO:	Nitric oxide
HIF:	Hypoxia inducible factor
VHL:	von Hippel-Lindau

bFGF:	Basic vascular endothelial growth factor
aFGF:	Acidic vascular endothelial growth factor
TGF-α:	Transforming growth factor
IL-1:	Interleukin-1
ECM:	Extracellular matrix
Gy:	Gray
RTOG:	Radiation Therapy Oncology Group
RCT:	Radiochemotherapy
AI:	Apoptotic index
CR:	Complete response
CDK2:	Cyclin-dependent kinase 2
TSG:	Tumour suppressor gene
LPS:	Lipopolysaccharide
MHC:	Major histocompatibility complex
GM-CSF:	Granulocyte-macrophage colony-stimulating factor
PAF:	Platelet-activating factor
TAM's:	Tumour associated macrophages
MI:	Macrophage index
APES:	3-aminopropyltriethoxysilane
DAB:	3,3-diaminobenzidine tetrahydrochloride
MCP-1:	monocyte chemotactic protein-1
CSF-I	Colony-stimulating factor

CHAPTER ONE

INTRODUCTION

INTRODUCTION

1.1. General consideration

260,000 new cancers (excluding non-melanoma skin cancers) were registered in the United Kingdom in 1997.

During 1997 10,480 new cases of bladder cancer were registered. These accounts for 4% of all cancers registered and make bladder cancer the fifth most common cancer in the United Kingdom. Only cancer of the lung (38,870 cases), cancer of the breast (38,270 cases), cancer of the colon and rectum (34,310 cases) and cancer of the prostate (21,777 cases) are more prevalent.

Bladder cancer is more common in men than in women, being the fourth most common cancer in men and the 8th most common in women. The difference in incidence between the sexes is becoming less and this may be related to the increased smoking habits seen in women.

There were 152,500 deaths due to cancer during 1999 and this accounts for 25% of all deaths. Figures collected between 1991 and 1993 indicate that the overall 5 year survival for bladder cancer in men was 65.7% and for women 57.6%. Thus during that period bladder cancer had the best 5 year survival for all common male cancers and the 4th best for common female cancers.

The figures above were taken from the Cancer Research Campaign website (www.crc.org.uk).

It is therefore clear that the commitment required for the management of bladder cancer involves a significant part of most urologists' time.

The majority (90%) of bladder cancers are transitional cell (TCC) cancers. Adenocarcinomas, squamous cell carcinomas and a variety of rare cancers make up the remainder (Barbara, *et al.*, 1996).

TCCs may present in a variety of histological grades and stages. The majority are well differentiated and superficial on the bladder wall. A minority (approximately 20%), however, are less well differentiated and invade into and through the muscular wall of the bladder. At the present time the only hope of cure with such tumours is either by total removal of the bladder (cystectomy) or external beam radiotherapy (EBRT). Although cystectomy is usually considered to have superior results as regards survival, the difference is small (5-year survival after cystectomy is 59% for pT2, 25% for pT3 and 29% for pT4) (Dalbagni, *et al.*, 2001) and radiotherapy has the advantage of organ preservation and avoiding surgery. Therefore, there would be a clinical advantage if parameters were available to select patients most likely to respond to radiotherapy.

It is recognised that tumour oxygenation (in part, related to tumour vascularity) is positively correlated with response to radiotherapy (Hopewell *et al.*, 1989). Tumour oxygenation requires a neovascular response to the tumour and there is considerable direct evidence to support the idea that tumour growth and metastasis require neovascularisation. The process by which this neovascularisation occurs is known as tumour angiogenesis (Folkman, 1990). Angiogenic stimulators, as well as angiogenic inhibitors tightly regulate new vessel growth. As tumour growth and tumour invasion depend on this angiogenic response the ability to quantitate the degree of angiogenesis within or around a tumour may provide important prognostic information (Weidner *et al.*, 1993; Pluda, 1997). Immunohistochemical methods have been developed which are capable of quantitating the neovascular response induced by a specific tumour. This quantitation has been accomplished by determining the microvessel density within and

around a tumour, using antibodies that recognise vascular endothelial cells (Stein *et al.*, 1998).

1.1.1 The aetiology of bladder cancer

Bladder cancer was the first cancer identified as being associated with industrialisation. In 1895, Dr. Ludwig Rehn reported on bladder cancer in German workers who manufactured aniline dyes. Later, this was shown to be related to the presence of 2-naphthyl-amine in the dyes. Since then, many additional chemicals, along with environmental agents, have been identified as causes of bladder cancer (Clayson *et al.*, 1970; Price, 1971; Cohen *et al.*, 1992).

Although a number of aetiological factors are associated with the development of bladder cancer, in industrialised countries cigarette smoking is by far the most significant environmental carcinogen today (Chiu *et al.*, 2001). A fourfold increased risk for the development of bladder cancer has been reported in cigarette smokers (Clavel *et al.*, 1989). The type and number of cigarettes, and use of filters have each been shown to be related, in a dose response manner, to the development of bladder cancer. Also time since cessation of smoking has been related to a reduction in its development (Vineis *et al.*, 1998).

Controversy exists as to whether coffee and other caffeine-containing beverages are involved in urinary tract carcinogenesis (Cohen *et al.*, 1992). The results of epidemiological studies show marked variation, with studies indicating either no risk or a slight increase in relative risk. Interpretation of the studies is often difficult because of the presence of confounding factors, especially cigarette smoking. Experimental studies have failed to demonstrate that caffeine is carcinogenic to the urinary bladder (Johansson, *et al.*, 1997). Also, both long-term carcinogenicity studies and multistage

studies have been negative for caffeine (Sternberg, 1999). The genotoxicity of caffeine also remains uncertain and is largely dependent on the type of assay being used and the caffeine concentrations attained in the assay system. The evidence strongly favours the conclusion that caffeine is not mutagenic in humans (Johansson, *et al.*, 1997).

Treatment with cytostatic drugs, especially cyclophosphamide, is associated with the risk of bladder cancer, as is the treatment of tissues lying close to the bladder with radiotherapy e.g. for uterine cancer (Tuttle *et al.*, 1988; Kleinerman *et al.*, 1995).

In developing countries, especially in the Middle East and parts of Africa, infections with organism Schistosoma Haematobium is responsible for a high incidence of bladder cancer although 75% of these cases are squamous cell carcinomas rather than transitional cell carcinoma (El-Bolkainy, 1983; Cohen *et al.*, 1992).

Certain areas of the Balkan countries, including the former Yugoslavia and Bulgaria, have increased death rates due to nephropathy. These patients also have a high prevalence of urothelial tumours involving both upper and lower urinary tracts. So far, extensive research has failed to reveal specific aetiologic factors responsible for the cancers in these patients. A mycotoxin and ochratoxin may induce nephropathy in pigs similar to the Balkan type in humans. It is unclear whether this compound is involved in the human disease or is related to the urothelial malignancies (Cohen *et al.*, 1992).

Certain regions of the Island of Taiwan have an increased incidence of peripheral vascular disease commonly referred to as "Blackfoot disease". Individuals suffering from this condition have an increased incidence of urinary tract malignancy (Johansson, *et al.*, 1997; Yang *et al.*, 2002). Arsenic has been suggested to be the culprit responsible for the vascular disease process and may also be involved in the development of urothelial cancer. A similar relationship between high levels of arsenic exposure and bladder cancer has been reported from Argentina and Chile (Hopenhayn-Rich, *et al.*, 1996).

Dietary factors in experimental animals and humans have been observed to influence the development of bladder cancer. It has been observed that individuals with greater vitamin A or carotene consumption have a lower incidence of bladder cancer than those with low consumption of the vitamin. To some extent, experimental studies have supported this view and have formed the basis for the use of retinoids as potential chemopreventive agents in patients with a previous papillary transitional cell carcinoma (Johansson, *et al.*, 1997; Ross *et al.*, 1996).

N-nitrosamines are chemicals that can produce bladder cancer in rodents (Magee *et al.*, 1967). Nitrosamines can be formed in vivo from ingested nitrates and secondary amines by nitrate-reducing bacteria in the human bladder. Vitamin C can block this in vivo nitrosamine formation, but there is little epidemiological information at present available on the relationship between N-nitroso compounds or vitamin C intact and bladder cancer risk (Ross *et al.*, 1996).

Exposure to drinking water in areas with a high pesticide usage is associated with an increased risk of development of bladder cancer (Cohen *et al.*, 1992; Lamm and Torti, 1996), although the reason for this is unclear.

There has been a suggestion that artificial sweeteners, especially sodium saccharin and cyclamate, are associated with increased risk of bladder cancer (Cohen *et al.*, 1992). A large number of epidemiological studies have been performed to evaluate the relationship between exposure to these sweeteners and the development of bladder cancer in humans but have failed as yet to find such a relationship.

Individuals who are immune-suppressed as a result of disease, such as genetic immunodeficiencies and acquired immunodeficiency syndrome (AIDS), or as a result of treatment, such as transplant patients, have an increased risk of developing malignancies.

Patients who receive a solid organ transplant have a threefold to fourfold increased risk of developing a malignancy (Penn, 2000). Three previous cases of bladder carcinoma after heart transplant have been reported (Maier and Grimm, 1994; Baldwin and Ruckle, 1995; Stein *et al.*, 1995). In these cases, the patients were diagnosed with bladder carcinoma 4 to 5 years after transplantation. The investigators of these reports speculated that chronic immunosuppression played a role in the development of the bladder carcinoma. In addition, 12 cases of bladder carcinoma in renal transplant patients have been also reported (Gifford *et al.*, 1998). In most resent report by Berger *et al.*, in 2002, reported the first case of transitional cell carcinoma of the bladder in a lung transplant recipient. Although, a direct causal relationship between the two cannot be established, the presentation of the tumour 1.5 years after lung transplantation suggests that chronic immunosuppression was a contributing factor.

The human papilloma virus (HPV) family has been implicated in the development of bladder cancer (Zur, 1996). The presence of HPV DNA in squamous and transitional cell carcinoma of the bladder has been detected in 0-80% of tumours examined (Griffiths *et al.*, 2000). Nevertheless, most studies report low HPV numbers (<10%) (Simoneau *et al.*, 1999; Soulitzis *et al.*, 2002), suggesting that HPV plays an important role in a small portion of bladder cancer cases.

Urinary tract calculi have also been associated with an increased risk of bladder cancer (Burin *et al.*, 1995; LaVecchia and Airoldi, 1999). This is likely to be due to repeated abrasion of the urothelium with regenerative hyperplasia and is frequently associated with squamous cell carcinoma rather than transitional cell carcinoma.

Advanced techniques based on the molecular biology of bladder cancer have detected chromosomal aberrations correlating directly to mechanisms of carcinogenesis and tumour progression. Karyotyping reveals numeric alterations of the chromosomes 7, 8, 9 and structural aberrations of the chromosomes 1, 5, 9, 11, 13 and 17, which finally, on

the molecular level, lead to alterations of the cellular activity and function of proto-oncogenes and tumour suppressor genes and to modulated cellular signaling and transcription. The loss of specific chromosomal regions in urothelial carcinoma can result in structurally altered and therefore inactivated tumour-suppressor genes or activated carcinogenic oncogenes (Brauers *et al.*, 2000).

Numerical chromosomal aberrations in urothelial carcinoma mostly affect chromosome 9 (Sandberg and Berger, 1994). A complete deletion of chromosome 9 is present in about 50% of all bladder cancer specimens, but is rarely the only cytogenetic event and, in the majority of cases, is accompanied by various structural and numerical alterations of other chromosomes (Sandberg and Berger, 1994).

Structural chromosomal aberrations in transitional cell carcinoma mostly affect chromosomes 9, 11, 17, 18, 3 and 5 (Brewster *et al.*, 1994; Sandberg and Berger, 1994). Several oncogenes and tumour suppressor genes, such as p16, p21 (WAF1/CIP1), Rb1, ras, c-erbB-1 and p53 are involved in the development and progression of bladder cancer (Brauers *et al.*, 2000).

1.1.2 The epidemiology of bladder cancer

Bladder cancer is one of the most common diseases treated by urologists. This is due to its high incidence (5th most common cancer in the United Kingdom) and its tendency to recur anywhere the urinary tract is covered with transitional cell epithelium.

Most bladder cancers in the UK and United States are transitional cell carcinomas. It is the sixth most common cause of death from malignant disease in men and the 10th most common cause in women in the UK, with a mortality rate of 126 and 59 per million population, respectively (Working Group on Urological Cancer, 1996) and in 1999

4850 bladder cancer deaths (4% all cancer deaths) were reported (crc website). In 1997 a total of 10,480 new bladder cancer cases was reported (crc website).

In the South-Western Region of England, carcinoma of the bladder also ranks fifth among malignancies, with a crude annual incidence rate of 30.8 per 100,000 for men and 11.5 per 100,000 for women (Thorne *et al.*, 1994). Between 1997-2007 the incidence is predicted to rise by 9% and the number of deaths by 10% (South and West Cancer Intelligence Unit, 1997). In the South and West, as nationally, there are clear upward incidence trends for both sexes (South and West Cancer Intelligence Unit, 1997).

The incidence of the disease has been increasing progressively since 1955.Yearly incidence continues to rise despite improved awareness of cancer-related carcinogens, both at home and in the workplace.

Bladder cancer is the fourth most commonly diagnosed malignancy in men and the eighth most commonly diagnosed malignancy in women in the USA. In 2000, approximately 50,000 new cases were diagnosed, and over 10,000 deaths were attributed to the disease (Metts *et al.*, 2000).

The median age at diagnosis is 64 years, and the disease is rarely diagnosed before the age of 40. As the society ages, it will become even more significant as the fourth leading cause of cancer death in men aged over 89 years (Hassen *et al.*, 2000).

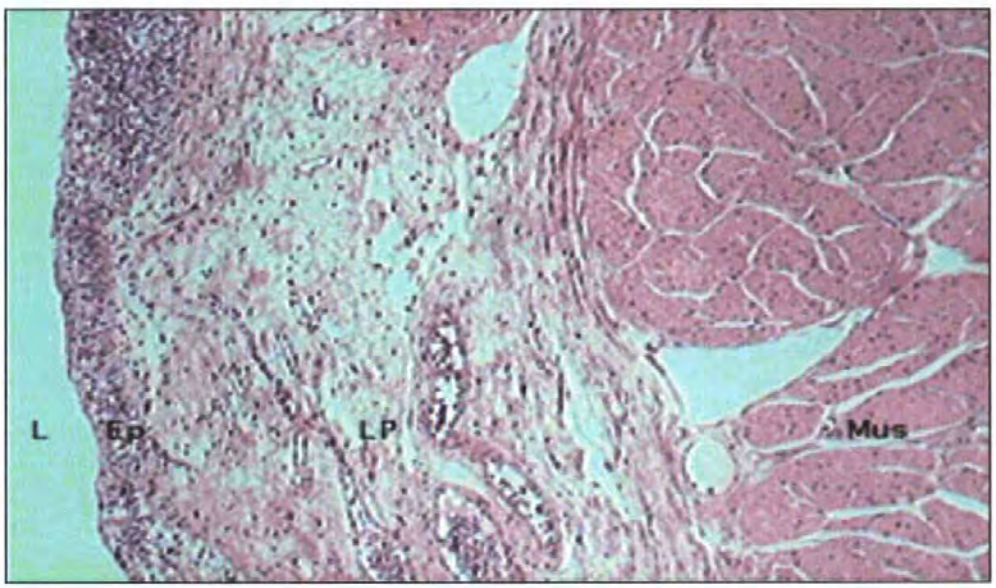
There is a marked male predominance in transitional cell carcinoma; the male to female ratio is at least 3:1 (Brauers *et al.*, 2000). However, recent reports have suggested an increased incidence of women presenting with muscle invasive cancer. Reasons for this trend are unclear but may be related to the increasing smoking habits in women (Hassen *et al.*, 2000).

1.1.3 The histopathology of bladder cancer

Familiarity with the histological and cytological features of the normal urinary bladder histology is essential to the understanding of the pathology of bladder cancer, leading to accurate tumour grading and staging that will ultimately determine the oncological management of these urothelial malignancies (Barbara, *et al.*, 1996). The wall of the urinary bladder (Fig. 1) consists of the mucosa (lining epithelium and lamina propria), muscularis mucosae and muscularis propria (detrusor muscle). At the bladder neck, the bladder is fixed by ligaments. The rest of the bladder is loosely contained by perivesical fat and on its superio-posteror aspect, the dome, it is covered by peritoneum.

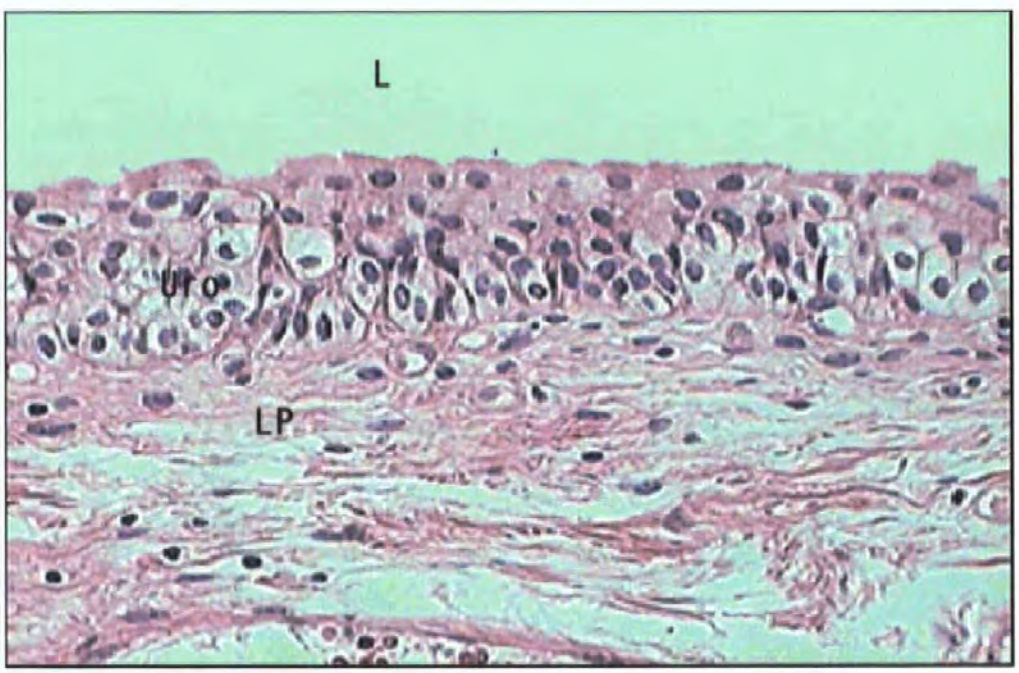
The urinary tract (bladder, ureters, and renal pelvis) is lined by a specialised transitional epithelium, the urothelium (Fig. 2), which protects the subepithelial tissue from endogenous and exogenous chemical and bacterial toxins. The normal urothelium is of variable thickness and the number of urothelial layers is dependent on the degree of organ distension. Normally, this epithelium ranges from three to seven cell layers in thickness. An increase in number is abnormal but not necessary neoplastic. The urothelium is composed of 2 distinct cell types, the superficial and underlying intermediate and basal cells. The superficial cell layer is a highly protective layer with large, and occasionally multinucleated specialized umbrella cells. They are modified to maintain the integrity of the mucosa during expansion and contraction. They have a rigid surface membrane, which maintains its shape even after exfoliation. Beneath the urothelial basement membrane is the lamina propria, consisting of loose connective tissue that contains delicate bundles of smooth muscle, the muscularis mucosae. The muscularis mucosae is a variably developed layer of thin smooth muscle fibres that may be complete, incomplete or attenuated (Reuter, 1992; Ro, *et al.*, 1987).

Fig. 1. Histology of the normal bladder.



L= Lumen, Ep= Epethelium (urothelium), LP= Lamina propria, Mus= muscularis propria.

Fig. 2. Transitional epithelium: epithelium cell layers



L= Lumen, Uro= urothelium, LP= Lamina propria.

Whether or not a muscularis mucosae is always found in the bladder is a contentious issue.

The wispy smooth muscle bundles of the muscularis mucosae are characteristically thinner than those of the muscularis propria, an important feature when assessing bladder biopsies. Also the muscularis mucosae is associated with large medium sized arterial and venous blood vessels. Both the lamina propria and submucosa usually consist of loose fibrous tissue, but may contain variable amounts of adipose tissue (Bochner, *et al.*, 1995), another potential source of diagnostic confusion.

The muscularis propria (detrusor muscle) consists of large bundles of muscle fibres associated with scant loose connective tissue. The arrangement of these bundles varies at different locations, for example they are more uniform and densely packed in the bladder neck, than elsewhere.

The urothelium shows a range of both non-neoplastic and neoplastic abnormalities, that should be accurately identified.

Von Brunn's nests (Fig. 3) contain well circumscribed urothelial aggregates that arise by a process of budding or migration of the overlying non-neoplastic urothelium. They may or may not be attached to the surface epithelium and are so common that they are considered as normal features of the bladder mucosa.

The term cystitis cystica (Fig. 4) is used to describe the histological feature of central degeneration of Von Brunn's nests, forming small cystic cavities lined by urothelium or cuboidal epithelium.

Cystitis glandularis is a similar entity that exists in 2 forms, the typical type and the intestinal type. It is composed of glandular structure within the lamina propria, that are lined by a large of cuboidal or columnar cells surrounded by urothelial cells. When the lining cells are overtly mucin-producing, the features are of the intestinal type of cystitis

Fig. 3. Von Brunn's nests:

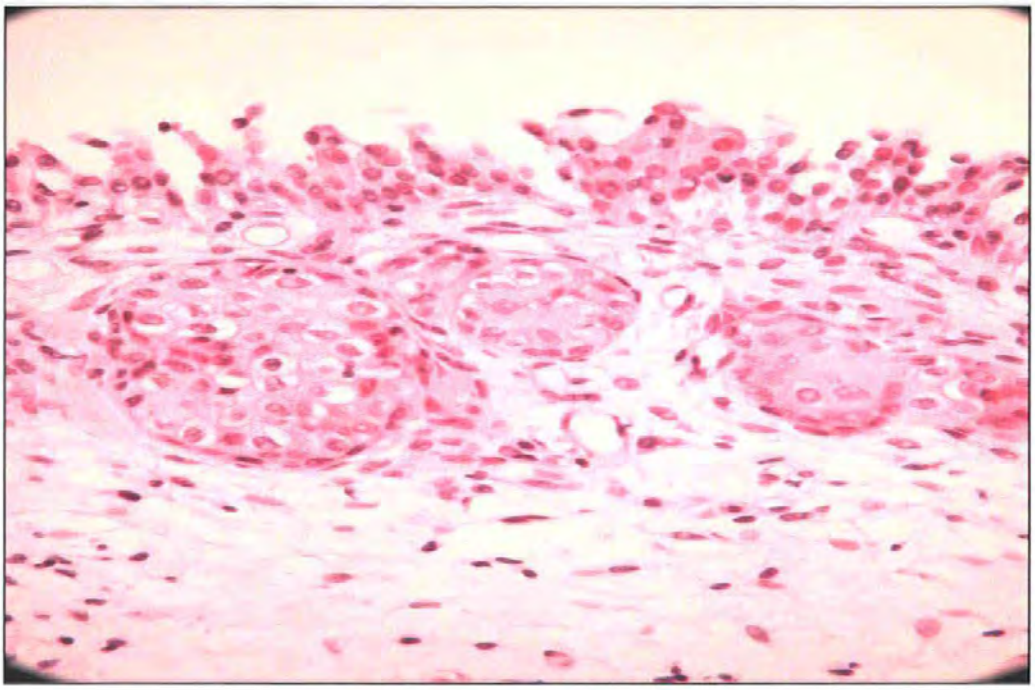
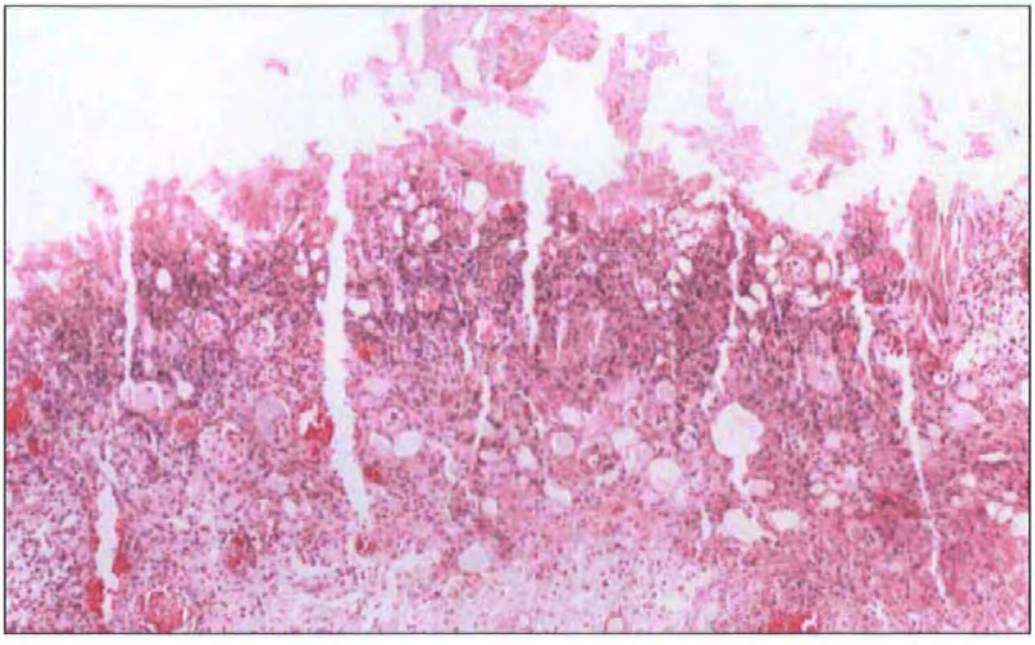


Fig. 4. Cystitis cystica:



glandularis. If this is diffuse it is known as intestinal metaplasia and is commonly seen in chronically irritated bladders, and this is associated with a risk of adenocarcinoma.

Approximately 95% of bladder tumours are of epithelial origin, the remainder being mesenchymal tumours (Table 1).

Neoplasms differentiating toward normal urothelium are called transitional cell neoplasms and these may arise any where along the urinary tract that is lined by urothelium.

In a series of about 1000 cases, the location of tumour within the bladder is listed as follows: lateral walls, 37%; posterior wall, 18%; trigone, 12%; bladder neck, 11%; ureteric orifices, 10%; dome, 8%; and anterior wall, 4% (Stephenson, *et al.*, 1990). They have also been reported within bladder diverticula and even arising from regenerated urothelium over a lyophilised dura patch (Selli, *et al.*, 1986). The pattern of growth of these tumours may be exophytic or endophytic, or a combination of both (Murphy, 1983). When exophytic, the tumour may adopt a papillary configuration (with a central fibrovascular cores) or a solid (nodular) appearance. When growing endophytically (especially if well-differentiated), it may result in nested formations in the lamina propria, which may be under diagnosed as von Brunn's nests or cystitis glandularis/cystica (Murphy, *et al.*, 1992; Talbert, 1989).

Classically TCC tumours are divided empirically into two categories, superficial and muscle invasive tumours. Confusingly, superficial tumours include those that have may have invaded lamina propria but not detrusor muscle whilst invasive tumours are those that have invaded into muscularis propria or beyond.

Superficial tumours account for approximately 80% of tumours at presentation (Abel, 1988; Metts *et al.*, 2000). They are generally amenable to bladder conservative therapy with disease controlled by transurethral resection (TUR), diathermy, laser and photodynamic therapy, intravesical chemotherapy and intravesical BCG.

Table 1. World health organization: Histological Classification of Urinary Bladder Tumours

Epithelial tumours	Nonepithelial tumours	Miscellaneous tumours	Metastatic tumours and secondary extensions	Unclassified tumours	Epithelial abnormalities	Tumourlike lesions
a. Transitional cell papilloma b. Transitional cell papilloma, inverted type c. Squamous cell papilloma d. Transitional cell carcinoma e. Variants of transitional cell carcinoma 1. With squamous metaplasia 2. With glandular metaplasia 3. With squamous and glandular metaplasia f. Squamous cell carcinoma g. Adenocarcinoma h. Undifferentiated carcinoma	a. Benign b. Malignant 1. Rhabdomy-Osarcoma 2. Others	a. Pheochromocytoma b. Lymphoma c. Carcinosarcoma d. Malignant melanoma e. Others			a. Papiloma (polypoid) cystitis b. Von Brunn's nests c. Cystitis cystica d. glandular metaplasia e. Nephrogenic adenoma f. Squamous metaplasia	a. Follicular cystitis b. Malakoplakia c. Amyloidosis d. Fibrous (fibroepithelial) polyp. e. Endometriosis. f. Cysts.

Only a small percentage of these evolve into tumours that invade the deep muscle and have the potential to metastasise (5% of Ta and 20% of T1).

The muscle invasive carcinomas account for approximately 15-20% of bladder tumours and require more aggressive treatment, either radical surgery or radiotherapy (Harland, 1994; Burchardt *et al.*, 2000). They are usually invasive at presentation, only 10-20% are preceded by lower stage tumours (Bostwick, 1992).

Clinical metastases are existent in approximately 5% of cases at presentation (Whitmore, *et al.*, 1988; Burchardt *et al.*, 2000). In these tumours the extent of invasion also influence treatment local extension beyond the bladder may lead to unresectability.

The most important histological features influencing treatment are the degree of tumour differention (grade) and the depth of invasion (stage). In particular the determination of the invasion of muscularis propria or beyond.

1.1.4 Tumour grade

No uniformly accepted grading system for bladder cancer exists. Most commonly the criteria of the 1973 World Health Organization are used (Mostofi, *et al.*, 1973), grading urothelial carcinomas into 1, 2 or 3.

Papilloma is a rare and high debatable entity. The controversy involves the existence of diagnostic criteria for this tumour. Some feel it would be better to extend this diagnosis to include the WHO gradel urothelial carcinomas (Fig. 5).

Grade 1 tumours have a thickened urothelium containing more than seven cell layers. The cells show mild nuclear pleomorphism and mitotic figures are rare. The nuclear polarity and architecture of the urothelium is retained. There is no necrosis (Fig. 6).

Grade 2 tumours are formed from urothelium that may be of increased, decreased or normal thickness. There is a greater degree of nuclear pleomorphism with scattered mitotic figures. The urothelial architecture is disturbed with loss of nuclear polarity. Necrosis is rare (Fig. 7).

Grade 3 tumours may have a solid growth pattern and are composed of highly pleomorphic cells with frequent bizarre mitotic figures (Friedell, *et al.*, 1980). There is architectural anarchy, often associated with necrosis (Fig. 8).

Within any given tumour there may be heterogeneity of grade, and the grade assigned should represent the highest grade present.

Fig. 5. Papilloma of the urinary bladder: **(a)** This benign tumour is composed of several papillary projections that consist of a core of connective tissue lined on the surface by transitional epithelium that does not differ significantly from the normal epithelium of the urinary bladder. **(b)** This papilla has a central vascular core which is lined with cells that do not exceed the thickness of the normal contracted urinary bladder.

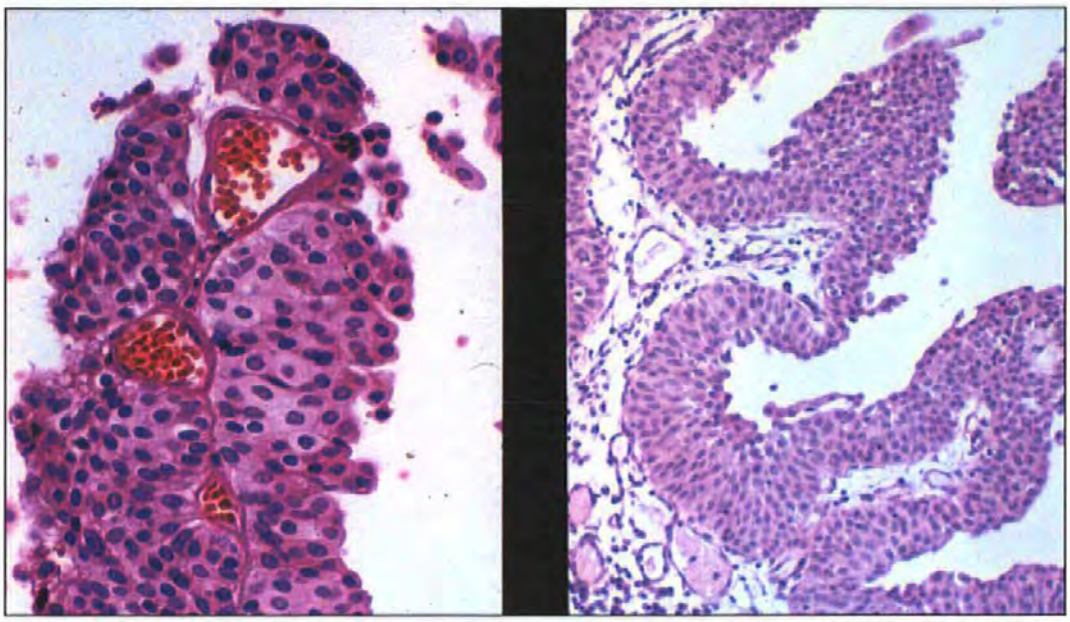


Fig. 6. Transitional cell carcinoma, grade I: thickened transitional cell epithelium that shows relative uniformity of cells and only mild loss of polarity.

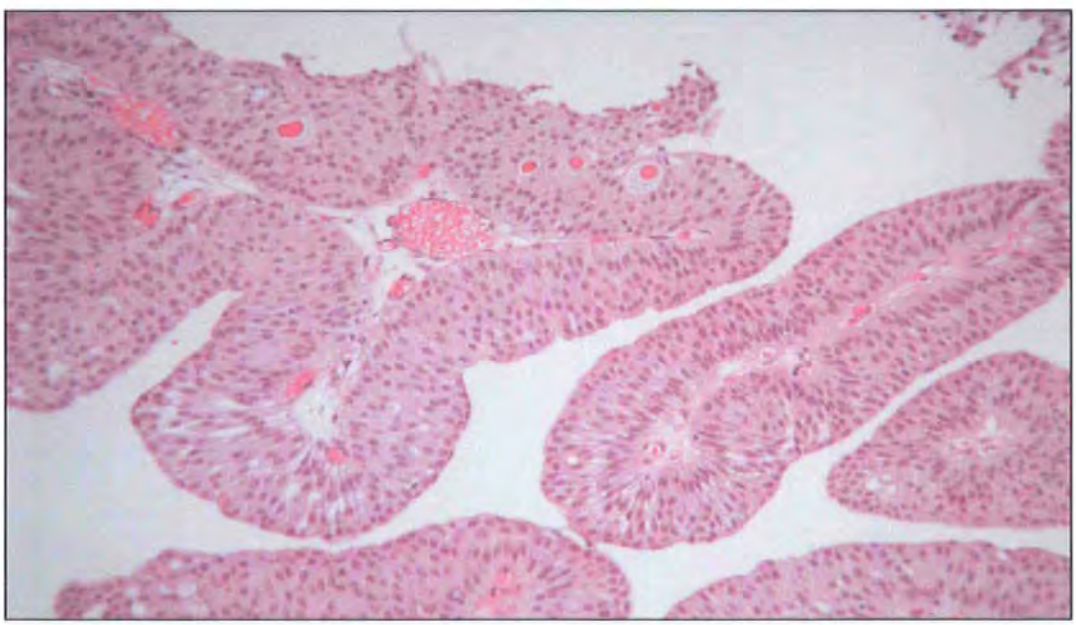


Fig. 7. Transitional cell carcinoma, grade II: The surface layer of this bladder tumour appears disorganized. The cells show variation in size and shape and the polarity of the nuclei has been lost.

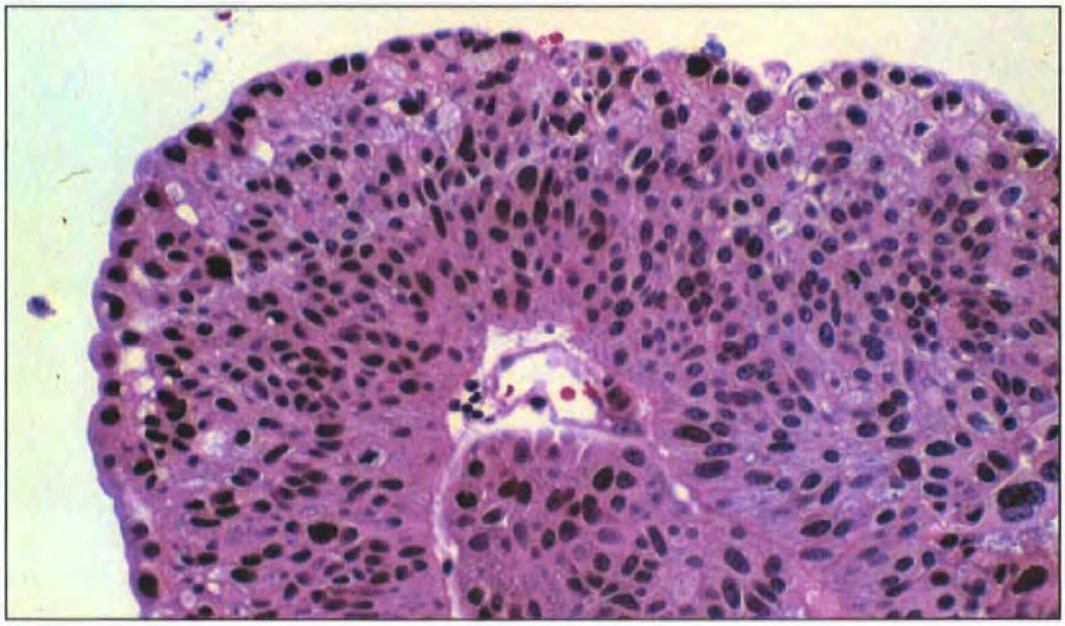
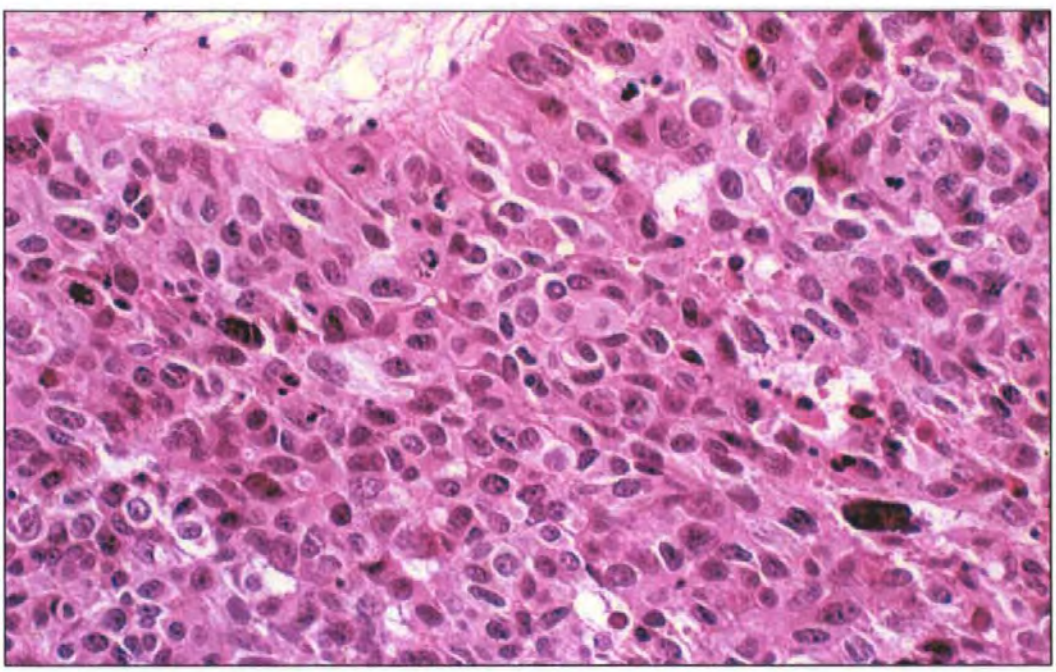


Fig. 8. Transitional cell carcinoma, grade III: The tumour is composed of cells that show marked pleomorphism, and only vaguely resemble transitional epithelium



1.1.5 Tumour stage

Tumour staging gives an indication of the extent of spread of the cancer and relies on information obtained from physical examination, radiological studies, endoscopy, examination under anaesthesia, biopsy, transurethral resection of the bladder tumour (TURBT), and other pertinent evaluations.

Clinical staging may be defined as an assessment of the extent of tumour invasion using clinical methods (including biopsy and TURB specimens), but not including definitive partial or total cystectomy material.

Pathological (surgical) staging is based on definitive surgical exploration and histopathological assessment of the extent of the primary tumour in a cystectomy specimen. Pathological staging of the tumour does not necessarily include full histopathological evaluation of the regional lymph nodes or potential sites of distant metastases.

The two most commonly used staging systems of bladder cancer include the Jewett and Strong system and the tumour-node-metastases (TNM) staging system.

The TNM system delineates exophytic and superficial growth patterns and also allows for separate classifications of patterns of spread outside the bladder (Table 2). Hence, it is more frequently used today (Sobin *et al.*, 1997).

Up to 80% of urothelial carcinomas are non invasive at presentation (pTa). The presence of stromal invasion (pT1) does have therapeutic and prognostic significance. It is debatable as to whether it is necessary to separate those invading the papillary cores only (pT1a) from those extending into the lamina propria (pT1b). However, microinvasion, defined as invasion of 5mm or less into the lamina propria was found to be associated with a significantly increased risk of metastatic cancer (Farrow and Utz, 1982). The importance of accurately assessing the depth of invasion cannot be underestimated.

Table 2. TNM Classification of Urinary Bladder Cancer:

The latest TNM system for staging bladder cancer was developed by the UICC in 1997

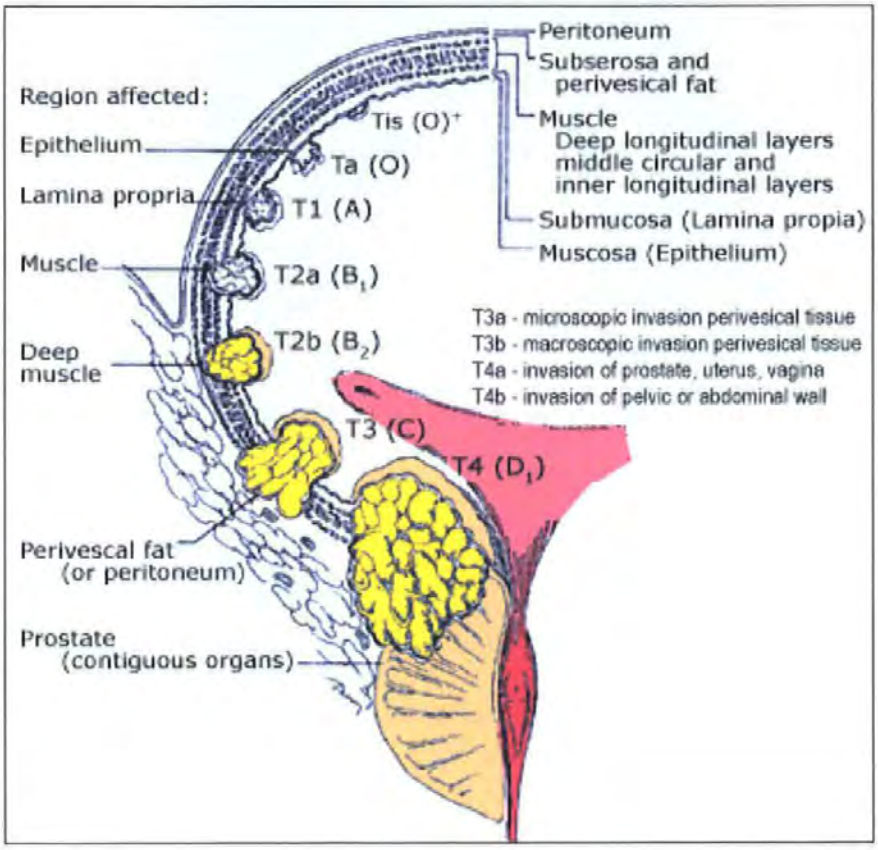
T - Tumour	N - Regional Lymph Nodes	M - Distant Metastasis
TX - Primary tumour cannot be evaluated **T0** - No primary tumour Ta - Noninvasive papillary carcinoma TIS - Carcinoma in situ ("flat tumour") T1 - Tumour invades connective tissue under the epithelium (surface layer) T2 - Tumour invades muscle **T2a** - Superficial muscle affected (inner half) **T2b** - Deep muscle affected (outer half) T3 - Tumour invades perivesical (around the bladder) fatty tissue **T3a** - microscopically **T3b** - macroscopically (e.g., visible tumour mass on the outer bladder tissue) T4 - Tumour invades any of the following: prostate, uterus, vagina, pelvic wall, abdominal wall	**NX** - Regional lymph nodes cannot be evaluated **N0** - No regional lymph node metastasis **N1** - Metastasis in a single lymph node < 2 cm in size **N2** - Metastasis in a single lymph node > 2 cm, but < 5 cm in size, or Multiple lymph nodes < 5 cm in size **N3** - Metastasis in a lymph node > 5 cm in size	**MX** - Distant metastasis cannot be evaluated **M0** - No distant metastasis **M1** - Distant metastasis

It is critical not to confuse the thin wispy muscle bundles of muscularis mucosae with those of detrusor muscle, as this will lead to overstaging of the tumour. Muscle invasion pT2 should only be diagnosed when tumour is seen invading thick muscle bandles. In TURBT specimens it is not possible to distinguish between invasion into the inner half of the muscularis propria (pT2a) and involvement of the outer half (pT2b). Fat may also been seen in between muscle bundles of the muscularis propria or within the lamine propria and submucosa and should not be confused with perivesical fatty tissue.

Analysis of treatment outcomes indicated that the most important determination is whether the tumour is organ-confined. To reflect the clinical importance of this, the classification has now changed. Pathologic T3 tumours are now defined as those that extend beyond the bladder to involve the perivesical fat, either microscopically (T3a), or macroscopically (T3b). It is believed that these changes will reflect survival differences found in patients with extravesical involvement by bladder tumours. Involvement of the prostate gland (pT4a) should be further defined as to whether it is confined to the prostatic urethra, involves the periurethral crypts or directly invades the prostatic tissue. Tumour fixed to the pelvic sidewall or invading the anterior abdominal wall is classified as T4b disease. These tumours are associated with the worst prognosis and cystectomy is not normally a treatment option. Figure 9 shows the relationship between the location and the depth of the tumour and the stage.

Carcinoma in situ (CIS) deserves special mention. CIS (Fig.10a&10b) is defined as high grade, flat, non-invasive, transitional cell carcinoma, often involving large portions of the bladder urothelium. CIS, although superficial by definition, tends to be aggressive, and may lead to metastatic disease (Metts *et al.*, 2000). It commonly occurs most often in association with bladder tumours of other types. Treatment depends on the extent of the tumour.

Fig. 9. The following illustration shows the relationship between the location and depth of the tumour and the stage.



Transurethral resection may be adequate in localized cases, but BCG and intravesical chemotherapy remain standard treatments. In patients with extensive CIS the risk of progression to invasive disease is up to 80% at 5 years (Utz and Farrow, 1984).

Risk of progression is related to grade and stage. The rate of progression for grade I is approximately 2%, 11% for grade II and 45% for grade III. Approximately 4% with Ta tumours progress, compared with 30% of patients with T1 tumours (Sternberg, 1999). The association of CIS with low stage, low grade tumours has been linked with increase risk of subsequent progression and muscle invasion. Up to 78% of patients with diffuse CIS, progress to muscle-invasive or metastatic disease (Dalbagni *et al.*, 2000). In muscle invading bladder cancers, there is a 50% risk of distant metastases (Sternberg, 1999).

Fig. 10a. Carcinoma in situ: The normal transitional epithelium has been replaced by thickened epithelium composed of cells that have hyperchromic nuclei and vary in size and shape. The nuclei show no polarization. These flat carcinoma-in-situ lesions are prone to endophytic growth and invasion of the bladder wall.

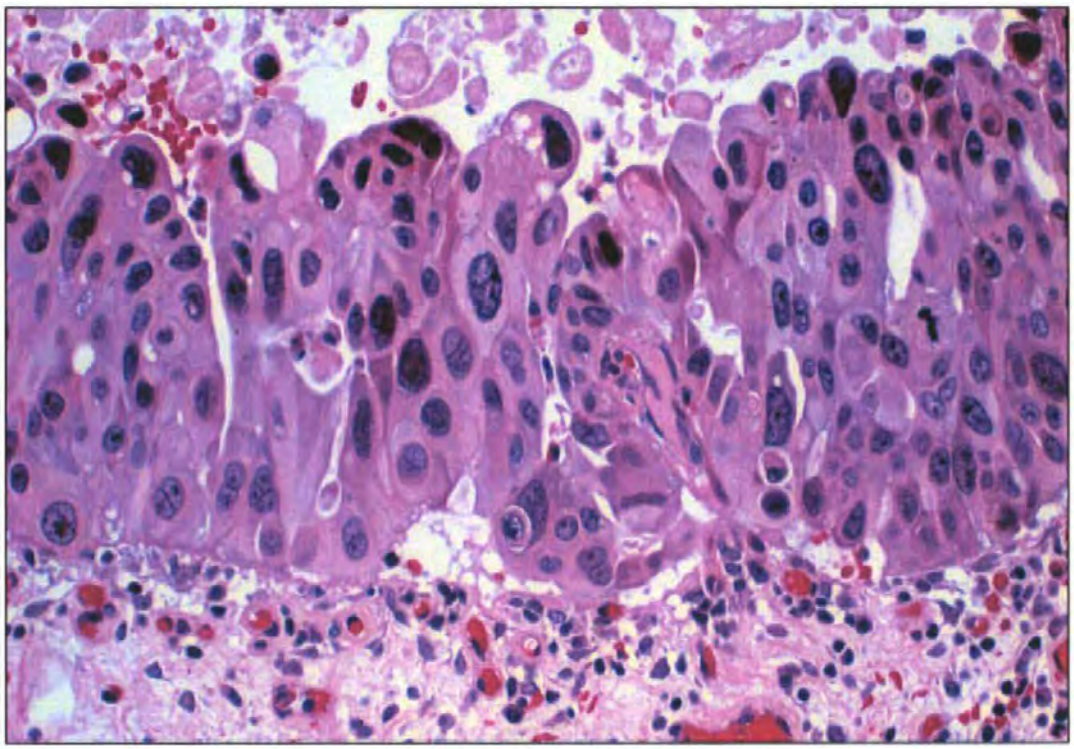
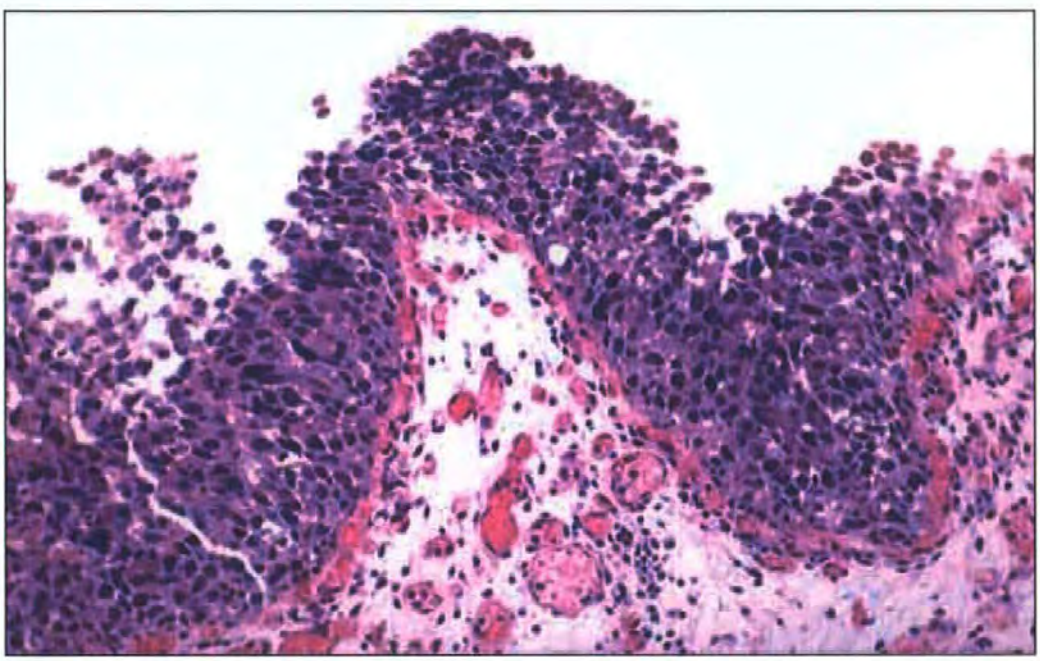


Figure 10b: Carcinoma in-situ. The nuclei are dark-staining and they vary markedly in their size and shape and involve the full thickness of the epithelium.



1.1.6 Diagnostic procedures

The most common presenting symptom of bladder cancer is painless haematuria occurring in 80-90% of cases (Varkarakis *et al.*, 1974). The symptom complex of bladder irritability (urinary frequency and urgency) and dysuria may occur and usually is associated with diffuse carcinoma in situ or invasive bladder cancer. The frequency may be due to sensory urgency, in which the bladder volume is normal, or in some cases due to an absolute reduction in the size of the bladder. Urinary infection is not uncommonly found in association with bladder cancer. Other signs and symptoms of bladder cancer include flank pain from ureteral obstruction, lower extremity oedema (as a result of lymphatic obstruction by the tumour), and a pelvic mass. Occasionally, patients present with symptoms of advanced disease, such as weight loss and abdominal or bone pain.

With the exception of young sexually active females with blood associated with a urinary infection, haematuria should be fully investigated. In patients under the age of 40 where the risk of malignancy is minimal, initial referral to a nephrologist is advisable.

Since transitional cells line the urinary tract starting at the kidney, down the ureter, into the bladder and includes most of the urethra, the entire urinary tract needs to be evaluated for transitional cell cancer.

Intravenous urography (IVU) should be performed to exclude upper tract obstruction and upper tract tumours. Larger bladder cancers may be seen as a filling defect on the bladder films but the sensitivity in detecting bladder cancer is poor and cystoscopy is always indicated.

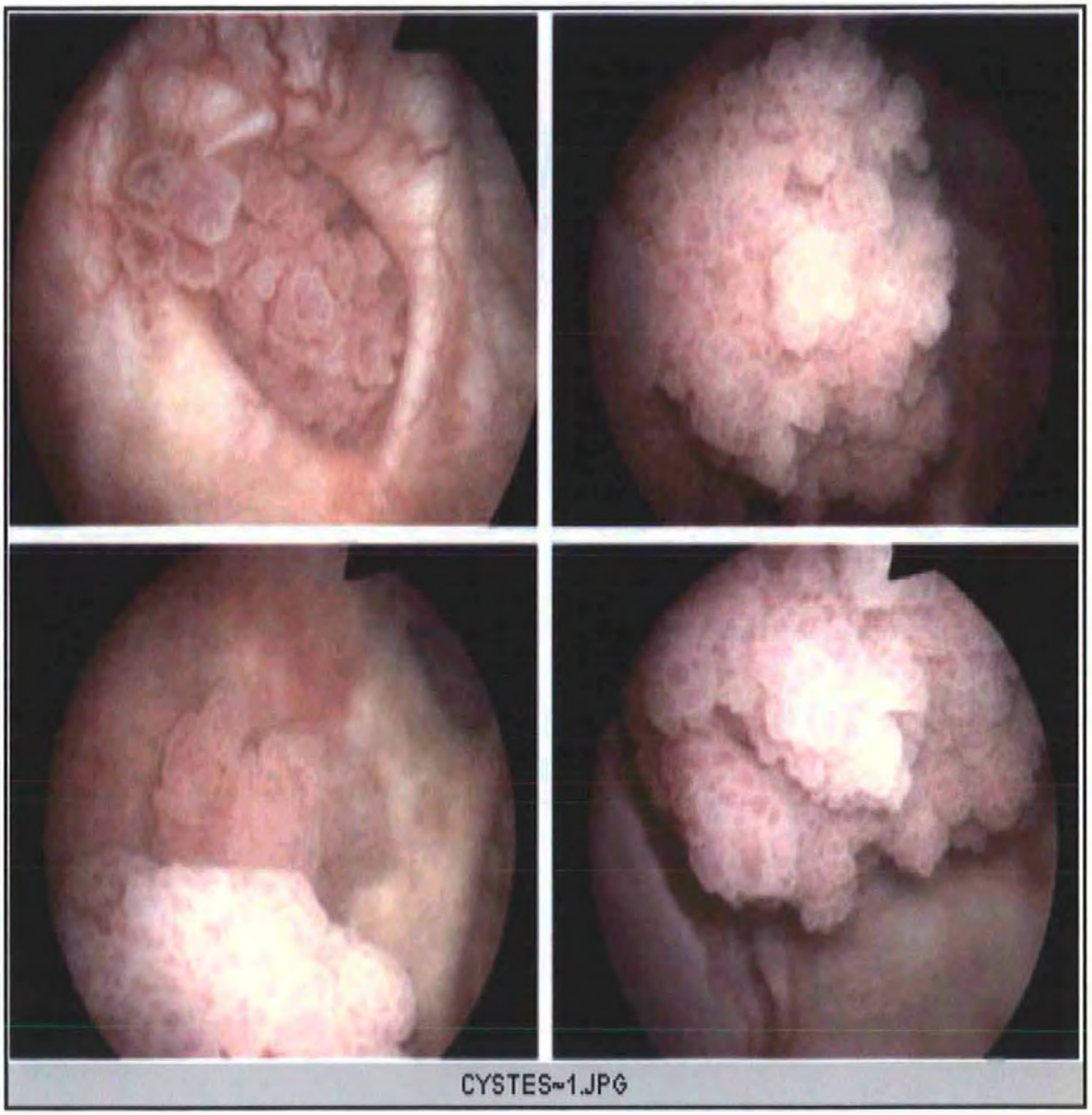
Ultrasound scanning of the upper tracts will also diagnose upper tract obstruction but has a lower sensitivity than IVU for diagnosing upper tract transitional cell tumours. It

should however be performed as it is more sensitive than IVU in detecting renal cell carcinoma.

Computerised tomography (CT) and magnetic resonance imaging (MRI) are generally not used in the initial diagnosis but are of value in staging tumours and planning prior to cystectomy and radiotherapy.

Cystoscopy is the definitive investigation at which mucosal abnormalities can be carefully documented and biopsies are taken (Fig. 11). If a bladder tumour is found it should be resected down to the bladder wall and separate biopsies of the base, including some detrusor muscle, taken in order that the pathologist can look for deep muscle invasion. At the end of resection a bimanual pelvic examination should be performed to enable clinical staging to be recorded.

Fig. 11. Surgeon's view of tumour (direct visualization of the tumour):



1.1.6.1 Urine cytology and other diagnostic tests

Since the tumours are bathed in urine attempts to diagnose a tumour non-invasively by means of urine analysis have been investigated. This would carry considerable benefits both for the patient who would avoid an uncomfortable operation, and would be cost effective for the purchasers of health care.

Voided urine cytology is the standard noninvasive tumour specific marker (Wiener *et al.*, 1993). Although in the hands of expert cytopathologists cytology is highly specific and sensitive for detecting carcinoma in situ, it is not sensitive for detecting low grade disease (Wiener *et al.*, 1993; Malik *et al.*, 1999). Furthermore, despite defined criteria for detecting malignant cells, variability among those interpreting cytology findings is significant. Some pathologists prefer a bladder wash (barbotage) specimen to a voided urine specimen to increase the yield of exfoliated cells. However, bladder wash is an invasive procedure and a fair number of cytopathologists discourage introducing instrumentation artifacts that may yield cells resembling low grade bladder cancer. These limitations force urine cytology to be only an adjunct to cystoscopy for detecting bladder cancer.

Wiener *et al.*, reported no difference in the sensitivity of cytology when barbotage or voided urine specimens were used (each 59% sensitivity) (Wiener *et al.*, 1998). The sensitivity rates in that study for detecting low to high grades 1 (17%), 2 (61%) and 3 (90%) bladder cancer emphasize that the sensitivity of cytology for detecting low grade bladder cancer is poor. Recently Konety *et al.*, reported that in patients without a history of bladder cancer the diagnostic value of bladder barbotage urine cytology is insignificant and, therefore, it is not cost-effective as part of a routine evaluation (Konety *et al.*, 1999). These results raise questions regarding the labor involved, procedure associated morbidity and the cost required to obtain bladder wash specimens. The specificity of urine cytology in several studies was 90% to 95% (Leyh *et al.*, 1999;

Takashi *et al.*, 1999; Pode *et al.*, 1999). Overall urine cytology is highly specific for detecting bladder cancer and yet except for high grade cancer detection it is not sensitive. Another problem with urine cytology is that it requires a trained cytopathologist. Since many clinicians do not have access to trained cytopathologists, many urologists do not have confidence in this diagnostic assay. Therefore, urine cytology in its present form cannot replace cystoscopy as a method for detecting and monitoring bladder cancer.

Over the past few years, a number of new tests have been devised to aid the diagnosis of bladder cancer. These tests include the bladder-tumour-associated antigen test (BTA™), the BTA stat test, the BTA TRAK test, the fibrin/fibrinogen degradation products test (FDP™), and the NMP22™ assay. All of these tests can be performed on urine samples.

Flow cytometry (FCM) measures the DNA content of cell nuclei and thereby gives an indication of the number of cells synthesized DNA and their proliferative activity. Melamed (1984) proposed that the presence of more than 15% aneuploid cells (compared to diploid) should define malignancy in the urothelium. In general, diploid tumours tend to be G1, Ta or T1 and tetraploid or aneuploid tumours are more likely to be poorly differentiated or invading muscle (Badalament *et al.*, 1987; Griffiths *et al.*, 1995; Tribukait *et al.*, 1982). Study carried out by Badalament *et al.*, showed in 228 patients that FCM varied according to T category; it was greater in TIS and T1 than in Ta. Comparison with cytology in a subset of 103 patients showed that the overall sensitivity of a single FCM examination was 78%, and that the sensitivity was significantly greater than voided urine cytology for all three categories of superficial bladder cancer. Cytology was positive when FCM was negative in 3/103 patients.

The nuclear matrix protein (NMP) 22 immunoassay measures a nuclear matrix protein released from urothelial cells into the urine. Nuclear matrix proteins determine nuclear morphology and regulate DNA replication and gene expression (Getzenberg *et al.*,

1997). NMP 22 has been developed as a quantitive test (Carpinito *et al.*, 1996; Miyanaga *et al.*, 1997), to predict the presence of TCC or the likehood of recurrence (Soloway *et al.*, 1996). Miyanaga *et al.*, found raised NMP 22 levels (>10.0 U/ml) in 81% (38/47) of patients with bladder cancer, compared with 36% (74/207) of patients following endoscopic resection of their tumour, or benign controls. Soloway *et al.*, reported that 86% of patients with post-transurethral resection levels less than 10U/ml had no recurrence at the first 3-month cystoscopy, compared with levels of NMP 22 greater than 10U/ml in post-transurethral resection urine that suggested a high risk of recurrence (Soloway *et al.*, 1996). These authors have suggested that this may prompt more aggressive management if NMP 22 levels are elevated following surgery. As a diagnostic test, NMP 22 was reported to have a sensitivity of 61% compared with 33% for voided urine cytology (Akaza *et al.*, 1997).

Several other studies have been done to determine the sensitivity and specificity of the NMP22 test. When evaluating these studies, it should be noted that although the manufacturer recommends 10 units per ml. as a cutoff limit for NMP22, others have used various cutoff limits of 3.6 to 12.0 units per ml. for calculating sensitivity and specificity. In these studies a wide range of sensitivity (47% to 100%) has been reported for NMP22 to detect bladder cancer but in many the value was between 60% and 70% (Wiener *et al.*, 1998; Leyh *et al.*, 1999; Ramakumar *et al.*, 1999; Sharma *et al.*, 1999; Banos *et al.*, 1998; Miyanaga *et al.*, 1999; Hughes *et al.*, 1999; Zippe *et al.*, 1999; Del Nero *et al.*, 1999; Witjes *et al.*, 1998). The NMP22 test appears to detect all grades of bladder cancer with similar sensitivity. In the studies of Del Nero and Landman *et al.*, NMP22 had overall 81% to 83% sensitivity for detecting bladder cancer, and 69% to 86%, 86% to 97% and 90% to 98% sensitivity for detecting grades 1 to 3 bladder cancer, respectively (Del Nero *et al.*, 1999; Landman *et al.*, 1998). In another study of Wiener et al NMP22 had an overall sensitivity of 48% (Wiener *et al.*, 1998). The

sensitivity rate for detecting grades 1 to 3 bladder cancer were 52%, 45% and 50%, respectively. In various studies the specificity of NMP22 is between 60% and 70%, although in one specificity was 90% (Landman *et al.*, 1998; Mahnert *et al.*, 1999; Heicappell *et al.*, 1999).

The bladder tumour antigen (BTA) test has been evaluated extensively. This is a latex agglutination test that detects the presence of basement membrane complexes in urine. Although more sensitive than cytology, the BTA test has been superseded by the BTA STAT test by the same manufacturers. This test measures complement factor H-related proteins in the urine (Kinders *et al.*, 1997). Leyh *et al.*, in his study found overall sensitivity of the new test for diagnosing bladder cancer to be 72% compared with 28% for cytology (Leyh *et al.*, 1997). Sarosdy *et al.*, compared BTA STAT with BTA in 181 archived urine samples and found superior sensitivity for BTA STAT (66% versus 59%) but equal specificity (Sarosdy *et al.*, 1997). In other studies the overall sensitivity of the BTA STAT was 57% to 83% (Wiener *et al.*, 1998; Leyh *et al.*, 1999; Sharma *et al.*, 1999). In 2 studies that evaluated the sensitivity of BTA STAT test by tumour grade sensitivity was 13% to 48%, 36% to 67% and 63% to 80% for detecting grades 1 to 3 disease, respectively (Leyh *et al.*, 1999; Gutierrez *et al.*, 1998; Nasuti *et al.*, 1999). Specificity of the BTA STAT in healthy individuals was reported to be 97%, whereas in patients with a history of bladder cancer it was 57% to 73%. It is important to note that the specificity of the BTA STAT in patients with benign genitourinary conditions, such as infection, renal disease, genitourinary trauma, cystitis, renal or bladder calculi, nephritis and renal stones, is only 46% (Leyh *et al.*, 1999). Furthermore, the BTA STAT has been shown to have an 84% false-positive rate in patients with signs and symptoms of dysuria, incontinence and haematuria (Nasuti *et al.*, 1999). It is not surprising that several genitourinary conditions that cause haematuria yield a false-positive result on the BTA STAT. In haematuria complement factor H in blood reacts with antihuman

complement factor H related protein monoclonal antibodies to yield a false-positive reaction on the BTA STAT.

The BTA TRAK test, which is a quantitative enzyme immunoassay using monoclonal antibodies to bind bladder tumour antigen in urine (Ishak *et al.*, 1997). Comparison with BTA in 108 archived samples showed improved sensitivity for BTA TRAK: sensitivity for grades 1, 2 and 3 tumours were 55%, 67% and 85%, compared with 41%, 58% and 70% for BTA.

In several studies the sensitivity of the BTA TRAK is 62% to 77% when using 14 units per ml. as the cutoff (Thomas *et al.*, 1999; Mahnert *et al.*, 1999; Abbate *et al.*, 1998; Irani *et al.*, 1999; Heicappell, *et al.*, 1999; Ellis *et al.*, 1997). Thomas *et al.*, reported that the sensitivity of the BTA TRAK for detecting grades 1 to 3 tumours was 48%, 59% and 88%, respectively. Sensitivity of the BTA TRAK varies considerably depending on the cutoff limit. Mahnert *et al.*, demonstrated that while the BTA TRAK had 62% sensitivity and 54% specificity for detecting bladder cancer at 14 units per ml. cutoff, it had only 13% sensitivity when the cutoff was 1,300 units per ml., at which point the specificity of the test for benign urological conditions was 95%. Overall specificity of the BTA TRAK in various studies was 48% to 70%.

Measurement of fibrinogen degradation products in urine is the basis of the Aura-Tek FDP dipstick rapid immunoassay. Two studies have shown the test to be significantly better than cytology for the detection of bladder cancer. Overal sensitivity of 69-84% and specificity of 78-79% compared with 33-39% sensitivity for cytology (Johnston *et al.*, 1997; Schmetter *et al.*, 1996).

Telomerase is a ribonucleoprotein enzyme that is present in almost all cancer cells but not in benign tissue (Lee *et al.*, 1997; Muller *et al.*, 1997). Telomerase can be measured in the urine or bladder washings (Kavaler *et al.*, 1997; Lance *et al.*, 1997) by a fluorescein-labelled PCR (polymerase chain reaction) based technique, or highly

sensitive telomeric repeat amplification protocol (TRAP assay) (Lance *et al.*, 1997; Muller *et al.*, 1997). Several studies have shown that telomerase is a promising marker for detecting bladder cancer. The overall sensitivity of telomerase for detecting bladder cancer is between 70% and 86% (Ramakumar *et al.*, 1999; Kavaler *et al.*, 1998; Lee *et al.*, 1998; Dalbagni *et al.*, 1997). Series using bladder wash specimens or combined results of bladder wash and voided urine have indicated that the sensitivity of telomerase for detecting bladder cancer is as high as 95% (Lee *et al.*, 1998). However, in other studies sensitivity has been as low as 46% (Dalbagni *et al.*, 1997). Although telomerase has high sensitivity for detecting all grades of bladder tumours, its sensitivity is much higher for detecting high grade disease. In various series the sensitivity of telomerase for detecting grades 1 to 3 bladder tumours was 56% to 79%, 72% to 85% and 85% to 100%, respectively (Landman *et al.*, 1998; Ramakumar *et al.*, 1999; Yokota *et al.*, 1998). The overall specificity of telomerase for bladder cancer detection is often between 60% and 70% (range 60% to 90%) (Ramakumar *et al.*, 1999; Landman *et al.*, 1998; Kavaler *et al.*, 1998; Yokota *et al.*, 1998; Lee *et al.*, 1998; Mayfield *et al.*, 1998). The majority of false-positive cases involve chronic or severe inflammatory disease (Mayfield *et al.*, 1998). This finding is not surprising since telomerase is expressed in proliferative cells, such as activated lymphocytes (Greider *et al.*, 1996).

There are a number of other potentially useful "markers" for bladder cancer diagnosis, including blood group antigens, inducers of immune response which may help to predict the invasive potential of surface tumours; and urine markers, such as M344 antigen, autocrine motility factors, glycosaminoglycans, scatter factor, and microsatellite analysis.

Lokeshwar and Soloway in a recent study concluded that currently noninvasive bladder cancer tests cannot replace cystoscopy, although some have shown a promise of being

clinically useful. One or a combination of these tests-markers may prove to be a "prostate specific antigen" for bladder cancer (Lokeshwar and Soloway, 2001).

1.1.6.2 Bladder cancer screening

If screening methods could detect bladder cancers destined to become muscle invading while they are still superficial (and therefore amenable to successful therapy), it is likely that a significant reduction in morbidity and mortality would result. Practically all bladder cancers will cause haematuria at some point, but haematuria is often intermittent, even when caused by serious disease. Repeated testing for haematuria is needed, and once one specimen is positive, further evaluation should be done to determine its cause. Screening for haematuria can be done by microscopic urinalysis or by using a chemical reagent strip (stix testing) to test for haemoglobin. Significant microscopic haematuria will be missed by microscopy unless a centrifuged urine sample is examined. Routine urine analysis in microbiological laboratories does not include centrifugation before microscopy. Conversely, "stix" testing (which test for both intact red blood cells and free haemoglobin) is so sensitive that it can be positive when an insignificant amount of blood is present.

Two screening studies of the general population using haematuria home testing have been published. Messing and colleagues and Britton and associates solicited from general-practice patient care rosters all middle-aged and elderly men residing in geographically defined regions [south central Wisconsin and Leeds, England] who were not believed to have urologic malignancies or other known causes of haematuria, and requested them to test their urine 10–14 times at home with reagent strips for haemoglobin (Messing *et al.*, 1992; Messing *et al.*, 1995; Britton *et al.*, 1989; Britton *et al.*, 1992). If the result was positive even once, the subjects were asked to undergo formal urologic evaluation including intravenous urography, cytology, and cystoscopy.

The two studies had similar findings. Fifteen to 20% of the screened populations had haematuria. Of those who completed the workup, 6–8% were found to have urothelial cancers. Overall, 1.2–1.3% of those screened had bladder cancer diagnosed. Neither study had a prospective, randomized control population.

The Wisconsin study looked at state tumour registry data to compare the screening participants' outcomes with those of an unscreened population. Additionally, pathology materials from all men screened in whom bladder cancer developed and from all men age over 50 years old in Wisconsin (not screened) in whom bladder cancer developed during 1 year of the study were compared by a referee pathologist. In both populations, roughly the same percentage had low-grade (grade 1, 2) superficial bladder cancers 56.8% unscreened, 52.4% screened). Approximately 45% of the cancers (43.2% unscreened, 47.6% screened) were high grade. The proportion of muscle-invasive tumours was significantly higher in the unscreened population: 23.9% of all cancer registry patients versus 4.9% of all screened cancers. By 24 months after diagnosis, 16.4% of the unscreened patients who developed bladder cancer had died of bladder cancer. In contrast, none of the 21 men participating in the screening study in whom bladder cancer was detected has died of bladder cancer after 4–9 years of follow up (Messing *et al.*, 1998). It would seem that screening allowed the successful diagnosis of cancers destined to become muscle invasive at preinvasive stages. Compared with other diseases for which screening has been accepted as beneficial and worth the expense (eg, mammography for breast cancer in postmenopausal women, fecal occult blood testing or colonoscopy for colorectal cancer, and blood pressure checks for hypertension), bladder cancer screening with chemical reagent strips appears to be quite cost-effective (Lawrence *et al.*, 1995). Because this was not a prospective, randomised, controlled study, certain biases must be considered (Messing *et al.*, 1989; Messing *et al.*, 1992; Messing *et al.*, 1995). Lead time bias (the interval between when it could be diagnosed

if you looked for it and when it is diagnosed because of symptoms) could have contributed to the decreased mortality seen in the screened group. The follow up time for the screened population was 4–9 years, though, as compared with 2 years for the unscreened population. Likewise, it is unlikely that in the screened population, more indolent tumours with longer preclinical durations were detected (length bias sampling), as the distributions of the grades of cancers detected were similar in both groups. The worse outcomes in the unscreened population could be explained if they received less effective therapy than the screeners. The unscreened patient outcomes, however, paralleled those reported in contemporary series of optimum treatment for similar tumour stages and grades, so inferior therapy in the control population does not appear to have been involved. The control group was not randomised, and its subjects may have differed from the screeners in terms of their health consciousness or other risk factors. This screening method has other problems as well. Although the test's sensitivity approaches 100% for cystoscopically detectable tumours (Messing *et al.*, 1992; Messing *et al.*, 1995; Messing *et al.*, 1990), the specificity is only 8% and the PPV (positive predictive values) is also only 8% (although the PPV is 11% for all malignancies and 33% for serious disease).

In summary, in the absence of a prospective randomized trial, the available information strongly suggests that screening shifts the diagnosis to an earlier noninvasive stage, with a resulting decrease in mortality. If other screening tests could be used to reduce the number of false-positive results (thereby reducing the number of negative workups), both cost effectiveness and public and physician acceptance would be enhanced even further.

1.1.7 The treatment of bladder cancer

1.1.7.1 Superficial disease (Ta; T1; CIS)

Although it is possible to remove Ta, T1 tumours surgically, 50-70% of patients have a recurrence within 1-2 years (Van der Meijden, 1998). To prevent this, patients may be treated with adjuvantly intravesical drugs. These drugs are instilled in the bladder as a watery solution, kept in the bladder for 1-2 hours, and then simply voided. Cytostatic agents such as thiotepa, adriamycin, mitomycin C, and epirubicin have been used, and during the past decade BCG has been one of the most effective drugs given intravesically (Harris *et al.*, 1997). During the past two decades the European Organisation for Research and Treatment of Cancer Genito-Urinary (EORTC-GU) group and the British Medical Research Council's working party have performed a series of randomised phase III studies investigating the prophylactic treatment of stage Ta, T1 bladder cancer after transurethral resection. Many studies have shown the advantage of adjuvant treatment after transurethral resection in decreasing the recurrence rate of bladder tumours or in prolonging the disease free interval of patients (Van der Meijden, 1998; Sternberg, 1999). The studies failed, however, to show the superiority of one agent over another, probably with the exception of BCG. There is also no evidence that adjuvant prophylactic treatment is of long-term benefit compared with transurethral resection alone in progression to muscle invasive disease and duration of survival.

BCG is the treatment of choice for CIS, producing complete response rate in 70% of cases. In one study, this complete response rate was increased to 87% with additional maintenance therapy. This consisted of 3 weekly treatments every 6 months for 3 years (Lamm, 1995). A recently published Southwest Oncology Group study examined 385

patients randomized to either maintenance therapy (weekly instillations for 3 weeks at 3 and 6 months, and then every 6 months) or observation after an initial 6 weeks BCG induction course (Lamm, *et al.*, 2000). The treated group showed greater freedom from recurrence (35% in the observation group compared with 76% in the maintenance group).

Appropriate therapy for superficial disease is based on the risk of recurrence or recurrence with progression in each individual patient. Patients with an initial episode of solitary low-grade Ta disease will usually be successfully treated by resection alone. Patients with multiple Ta tumours, who are more likely to experience recurrence, may benefit from adjunctive intravesical chemotherapy. Those with T1 tumours, CIS alone or CIS in the presence of papillary disease may be at a substantially increased risk for recurrence with progression. They may benefit from BCG therapy, but also should be considered for more aggressive surgical intervention if they do not demonstrate a prompt and complete response to conservative measures (Hassen *et al.*, 2000).

1.1.7.2 Muscle invasive disease (T2; T3; T4)

The therapies for muscle-infiltrating disease can be divided into those that are bladder sparing and those that are non-bladder-sparing (Table 3). The standard treatments with curative intent are surgical removal of the entire organ by radical cystectomy or external beam radiotherapy (EBRT). The latter can be given as a radical dose with curative intent or a smaller dose for palliation.

In appropriately selected cases of T2 disease, an aggressive TUR may be adequate, although this is controversial (Solsona, *et al.*, 1992). The key point is that neither approach is particularly successful as the very best 5 year survival rates that might be expected are 60-70% for T2 tumours, 50% for T3a tumours, 25% for T3b tumours and

Table 3. Treatment Options for Muscle-Infiltrating Disease:

Treatment options	Bladder sparing	Non-bladder sparing
TUR alone	Y	N
Neodymium YAG Laser	Y	N
Radiotherapy alone	Y	N
External beam	Y	N
Implantation	Y	N
Intraoperative	Y	N
Partial cystectomy	Y	N
Chemotherapy alone	Y	N
Chemotherapy + radiation	Y	N
Concurrent	Y	N
Alternating	Y	N
Sequential	Y	N
Radical surgery	N	Y
Chemotherapy + surgery	N	Y
Radiation + surgery	N	Y
Chemotherapy + radiation + surgery	N	Y

Y= Yes

N= No

only 5-10% for T4 tumours (Bloom *et al.*, 1982; Esring *et al.*, 1994). The most recent published data is from the University of Southern California. In this series the 5-year survival after cystectomy in 633 patients with pT2, pT3a, pT3b and pT4 disease was 72%, 58%, 38% and 33% respectively (Stein *et al.*, 2001). In 284 patients at the Memorial Sloan Kettering Cancer Center, 5-year survival with pT2 tumours was 59%, pT3 was 25%, and pT4 was 29% (Dalbagni *et al.*, 2001).

The exact value of radical radiotherapy is difficult to establish because changes in treatment techniques and selection of patients have biased the results. The 5-year survival rates are reported to be 35-71% in T1 tumours, 27-59% in T2 tumours, 10-38% in T3 tumours and 0-16% in T4 tumours (Sengelφv *et al.*, 1999).

Full dose external-beam irradiation is a common treatment scheme for T2 and T3 tumours in Great Britain, with cystectomy reserved for salvage therapy. The largest radiotherapy series, with long-term follow up, is the Edinburgh series.

With a minimum dose of 55 Gy in 4 weeks, 25% of 5-year local control was achieved for T1, T2 and T3 patients; in T4, the long-term local control was 16% (De Braud *et al.*, 2002).

Cystectomy alone has not been tested against definitive radiotherapy in randomized trials (Dunst *et al.*, 2001). However, four relatively small trials compared preoperative radiotherapy and planned cystectomy vs. definitive radiotherapy alone with selective salvage cystectomy (Table 4).

The smallest study, from the M. D. Anderson Cancer Center, showed a benefit for radical cystectomy; however, the surgical patients in this study were pretreated with a potentially curative preoperative radiation regimen with 50 Gy (Miller *et al.*, 1977). The other studies demonstrated comparable results with preoperative radiotherapy and planned cystectomy vs. radical radiotherapy with salvage cystectomy (Horwich *et al.*, 1995; Sell *et al.*, 1991).

Table 4. Randomized Trials of Preoperative Irradiation and Planned Cystectomy vs. Radical Radiotherapy Alone in Muscle- Invading Bladder Cancers

Treatment	No. of patients	Stage	5-year overall survival
M.D. Anderson Hospital (Miller et al.,1977)			
50 Gy + cystectomy	35	T3	46%
60 Gy (+salvage cystectomy)	32	T3	22%
U.K. Co-op Group (Horwich et al., 1995)			
40 Gy + radical cystectomy	98	T3	39%
60 Gy (+ salvage cystectomy)	91	T3	28%
National Danish Trial (Sell et al.,1991)			
40 Gy + radical cystectomy	88	T3	29%
60 Gy (+ salvage cystectomy)	95	T3	23%
National Bladder Cancer Group #			
40 Gy + radical cystectomy	37	T2-T4a	27%
60 Gy (+ salvage cystectomy)	35	T2-T4a	40%

S. D. Cutler, personal communication 1983.

In summary, a significant advantage of cystectomy has not yet been proven (Dunst *et al.*, 2001).

Metastatic bladder cancer is usually incurable and the general approach is to help palliate symptoms. Although some patients with minimal metastatic disease may benefit from aggressive treatment, the vast majority of patients with metastatic disease should be managed with palliative intent. Individualized treatment plans are required, because each patient differs in his or her needs. Radiation can help alleviate many symptoms and chemotherapy may be offered as well. Radical surgery is not usually warranted in these cases (Metts *et al.*, 2000).

Randomized trials have demonstrated that M-VAC (methotrexate, vinblastine, doxorubicin and cisplatin) is the most effective chemotherapeutic regimen (Perry *et al.*, 1994; Scher, 1992). For the M-VAC regimen, various studies have shown response rates 30-50% (Loehrer *et al.*, 1992; Sternberg *et al.*, 1988). Unfortunately, M-VAC has substantial side effects and produces long-term survival in only, approximately 15-20% of patients.

The median survival duration is only 13 months and long-term survival is attained in, approximately 15% of patients with metastases in visceral sites and 30% of those with nodal disease (Oosterlinck *et al.*, 2002). Novel chemotherapeutic agents such as gemcitabine and the taxanes obtain similar overall survival, time to progressive disease, time to treatment failure, and response rate but gemcitabine + cisplatinum appears to have a reduced toxicity profile compared to M-VAC (Sternberg, 2000; von der Maase *et al.*, 2000).

The combination of gemcitabine and taxol has been shown to be highly effective in patients who have failed prior M-VAC (Sternberg *et al.*, 2000). When cisplatin gemcitabine and taxol were given to untreated patients, high overall response rate were observed (Bellmunt *et al.*, 2000).

The development of agents with activity in TCC has expanded the opportunity to combine them into synergistic and possibly more effective combinations. Recent clinical trials have placed an emphasis on defining the nature of response and toxicity to all possible combinations while recognizing that quality of life is an important endpoint in the treatment of patients with metastatic cancer (Table 5).

Two key problems remain: firstly, the accurate prediction of prognosis for bladder tumours, and secondly, the need for more effective treatments for all stages of disease. The problem of accurately assigning a prognosis to a particular tumour has led to attempts to identify clinico-pathological correlates which predict invasive potential, and might thereby allow a much more systematic approach to treatment, with earlier aggressive treatment of selected superficial tumours which are deemed likely to progress. In addition, a better understanding of the biology, and mechanisms of recurrence and progression in bladder cancer will help the development of new generations of rational therapeutic targets. A third and equally important goal of assigning an accurate prognosis to particular tumours is that patients with a dismal prognosis might be spared flawed attempts at affecting a cure through heroic but ultimately ill-advised surgery.

In 90% to 95% of cases, removal of the entire bladder is required to achieve local control. The exact indication may vary between institutions. Most physicians recommend cystectomy for (1) muscle-invading tumours (2) low-stage tumours unsuitable for conservative management, for example, because of multicentric and frequent recurrences resistant to intravesical instillation's, (3) high-grade tumours (T1G3) associated with Tis, or (4) bladder symptoms such as frequency or haemorrhage, rendering the patient a bladder "cripple". Survival distributions would be expected to vary for patients who undergo treatment for these indications.

Table 5. Combination regimens of new agents in metastatic TCC.

Study	Number of patients	Objective response rate %	Complete response rate %	Regimen
von der Maase et al (1997)	44	41	4	Gemcitabine + cisplatin
Stadler et al (1997)	47	59	11	
Moore et al (1999)	17	71	23	
Murphy et al (1996)	20	65	20	Paclitaxel + cisplatin
Burch et al (1999)	29	72	34	
Vaughn et al (1998)	33	50	12	Paclitaxel + carboplatin
Pycha et al (1999)	32	71	31	
Sengelov et al (1998)	25	60	28	Docetaxel + cisplatin
Garcia del Muro et al (1999)	19	53	21	
Tu et al (1995)	25	40	0	Paclitaxel + cisplatin + methotrexate
Oh et al (1999)	24	67	7	Cisplatin + 5-fluorouracil + Methotrexate
Bajorin et al (2000) and McCaffrey et al (1999)	44	68	23	Cisplatin + paclitaxel + ifosfamide
Meluch et al (1999)	26	60	8	Gemcitabine + paclitaxel

1.1.8 Chemotherapy

Chemotherapy given before cystectomy or definitive radiotherapy is termed neoadjuvant chemotherapy whereas that given after radical treatment is termed adjuvant chemotherapy. Many chemotherapeutic regimens have been tried in attempts to improve the outlook but with limited success. The most successful regimens include cisplatinum, methotrexate, vinblastine, and adriamycin (MVCA) and encouraging results have been obtained (Sternberg *et al.*, 1995).

1.1.8.1 Neoadjuvant chemotherapy

Neo-adjuvant chemotherapy is given before cystectomy or in some instances before radiation therapy. There are two principal reasons to use neo-adjuvant chemotherapy: to improve survival in patients with micrometastatic disease, and secondly to preserve the bladder (Sternberg *et al.*, 2000; Sternberg *et al.*, 1995). This approach has been useful in the treatment of several solid tumours. Although combination treatments with cisplatin, methotrexate, vinblastine, and adriamycin achieved complete remission in some patients, their impact on a large proportion of patients with bladder cancer remained unclear. Randomised prospective phase III trials comprising 100-300 patients were not able to detect a difference between definitive treatment alone versus neoadjuvant chemotherapy followed by definitive treatment. This may lead to the conclusion that neoadjuvant chemotherapy does not improve the prognosis of patients or that the difference might be so small that it cannot be detected in a series with limited numbers of patients. On the basis of the expected proportion of response from previous studies, the EORTC-GU group and the British Medical Research Council working party embarked on an international phase III study of neoadjuvant cisplatin, methotrexate, and vinblastine, and definitive local treatment with radical cystectomy or radiotherapy (Hall,

1996). Other international oncology groups have also joined the study. A total of 975 patients with muscle invasive bladder tumours and without detectable metastases were randomised. Patients received either three cycles of cisplatin, methotrexate, and vinblastine or no chemotherapy before radical cystectomy or full dose external beam radiotherapy. The study aimed to detect an absolute increase of three year survival of at least 10% in the chemotherapy arm. After a median follow up of 22 months the overall survival for patients treated with the drug combination was 62%, and 60% for those not receiving such treatment ($p=0.63$). As the follow up period is too limited, no definitive conclusion can be made yet. Apparently, however, if there is a difference in survival between the two groups it will be small. The results of this largest international series indicate that it is necessary to organise large prospective randomised studies to detect small differences, if any, between different treatments. Collaboration between trial organisations is therefore essential. Small series will not provide reliable conclusions, and patients might be harmed and efforts and funds wasted.

Table 6 displays the results of randomised neo-adjuvant chemotherapy trials in the literature. Many trials have a similar design; the differences between trials are due to the use of different types of chemotherapy.

Some of the trials have used single agent cisplatin, and some have used combination therapy. Although the equivalence of radiotherapy, cystectomy or a combination of both has not been proved by a randomised trial, all of these are used as local definitive treatment for muscle-invasive bladder cancer in several countries. There is no reason to expect that a benefit from chemotherapy would differ greatly with different local treatments. It seems that most of these trials appear to show no difference, but they may not have enrolled sufficient numbers of patients to detect realistic differences in survival.

Table 6. Randomised phase III trials of neo-adjuvant chemotherapy:

Study group	Neo-adjuvant arm	Standard arm	Patients	Results
Aust/UK (Wallace *et al.*, 1991)	DDP/RT	RT	255	No difference
Spain (CUETO) (Martinez *et al.*, 1995)	DDP/Cyst	Cyst	121	No difference
Canada/NCI (Coppin *et al.*, 1996)	DDP/RT or preop RT+Cyst	RT or preop RT+Cyst	99	No difference
EORTC/MRC (1999)	CMV/RT or Cyst	RT or Cyst	976	No difference
SWOG Intergroup (Natale *et al.*, 2001)	M-VAC/Cyst	Cyst	307	No difference
Italy (GUONE) (Bassi *et a.*, 1998)	M-VAC/Cyst	Cyst	206	No difference
Italy (GISTV) (1996)	M-VEC/Cyst	Cyst	171	No difference
Genoa (Orsatti *et al.*, 1995)	DDP/5-FU/RT/Cyst	Cyst	104	No difference
Nordic 1 (Malmstrom *et al.*, 1996)	ADM/DDP/RT /Cyst	RT/Cyst	311	No difference, 15% benefit with ADM/DDP in T3-T4a
Nordic 2 (Malmstrom *et al.*, 1999)	MTX/DDP/Cyst	Cyst	317	No difference
Abol-Enein (1997)	CarboMV/Cyst	Cyst	194	Benefit with CarboMV

DDP or C, cisplatin; MTX, methotrexate; ADM, doxorubicin; E, epirubicin; V, vinblastine; Carbo, carboplatin; Cyst, cystectomy; RT, radiation therapy; 5-FU, 5-.uorouracil; preop, preoperatively; M-VEC, methotrexate, vinblastine, epirubicin, cisplatin; M-VAC, methotrexate, vinblastine, doxorubicin, cisplatin; Aust/UK, Australia/United Kingdom; NCI, National Cancer Institute; EORTC/MRC, European Organization for Research and Treatment of Cancer/Medical Research Council; SWOG, South West Oncology Group.

1.1.8.2 Adjuvant chemotherapy

Adjuvant chemotherapy is given after cystectomy to patients at high risk of relapse (Sternberg *et al.*, 2000; Sternberg *et al.*, 1995). It is also widely used in patients with pT3-pT4a and/or pN+ M0 disease in an effort to delay recurrence and prolong survival. This approach of giving chemotherapy after local treatment has led to increases in survival in patients with several solid tumours. However, the advantage of adjuvant chemotherapy after radical cystectomy or radiotherapy has not been proved (Sternberg *et al.*, 1995). Studies have been hampered by small series of patients, patient selection, and high morbidity in elderly patients that have been exposed to extensive surgical procedures. In selected patients with minimal disease, adjuvant chemotherapy may improve survival (Skinner *et al.*, 1991) but conclusive evidence and large series are lacking.

The rationale for giving adjuvant (rather than neoadjuvant) chemotherapy is that the local definitive treatment is performed immediately. There is no delay in surgery and no time is wasted especially for those patients who do not respond to chemotherapy. Treatment decisions are based on pathological criteria, after careful examination of the cystectomy specimen. Micrometastases are treated when really at a low volume. Orthotopic bladder substitutions and the decreased morbidity of cystectomy are reasons to perform cystectomy and adjuvant chemotherapy (Sternberg, 2002).

The major disadvantage of treatment with adjuvant chemotherapy is the delay in giving systemic therapy for occult metastases while treating the primary tumour. Response cannot be easily evaluated, and the only clinical endpoint that can be assessed is time to recurrence. An additional disadvantage may be that it is more difficult to administer chemotherapy following cystectomy (Sternberg, 2002). There have been very few randomised trials evaluating adjuvant chemotherapy (Table 7).

Table 7. Trials of adjuvant chemotherapy following cystectomy

Investigator	Chemo	Chemo	No Chemo	Randomised	Results
Logothetis (1988)	CISCA	62	71	No	Benefit, but not randomised
Skinner (1991)	CAP	47	44	Yes	Benefit, but too few patients received therapy
Stockle (1992)	M-VAC/M-VEC	23	26	Yes	Benefit, small patient numbers, premature closure, no treatment at relapse
Studer (1994)	DDP	40	37	Yes	No benefit, single agent therapy probably inadequate
Bono (1995)	CM	48	35	Yes	No benefit for N0M0
Freiha (1996)	CMV	25	25	Yes	Benefit in relapse-free survival
Otto (2001)	M-VEC	55	53	Yes	No benefit

Chemo, chemotherapy; M, methotrexate; C, cisplatin; V, vinblastine; DDP, cisplatin; M-VEC, methotrexate, vinblastine, epirubicin, cisplatin; M-VAC, methotrexate, vinblastine, doxorubicin, cisplatin.

New trials should be developed on the basis of current knowledge. Urologists, medical oncologists, and radiotherapists should collaborate in providing more extensive evidence rather than relying on the limited results of single studies.

Treatment considerations should include a cost benefit analysis. In most countries, three to four cycles of chemotherapy cost (at 1998 prices) the equivalent of about £3000, radiotherapy costs about £1500, and radical cystectomy including hospital stay costs about £6000. Such economic considerations may play an increasingly important role in the future treatment of bladder cancer (Van der Meijden, 1998).

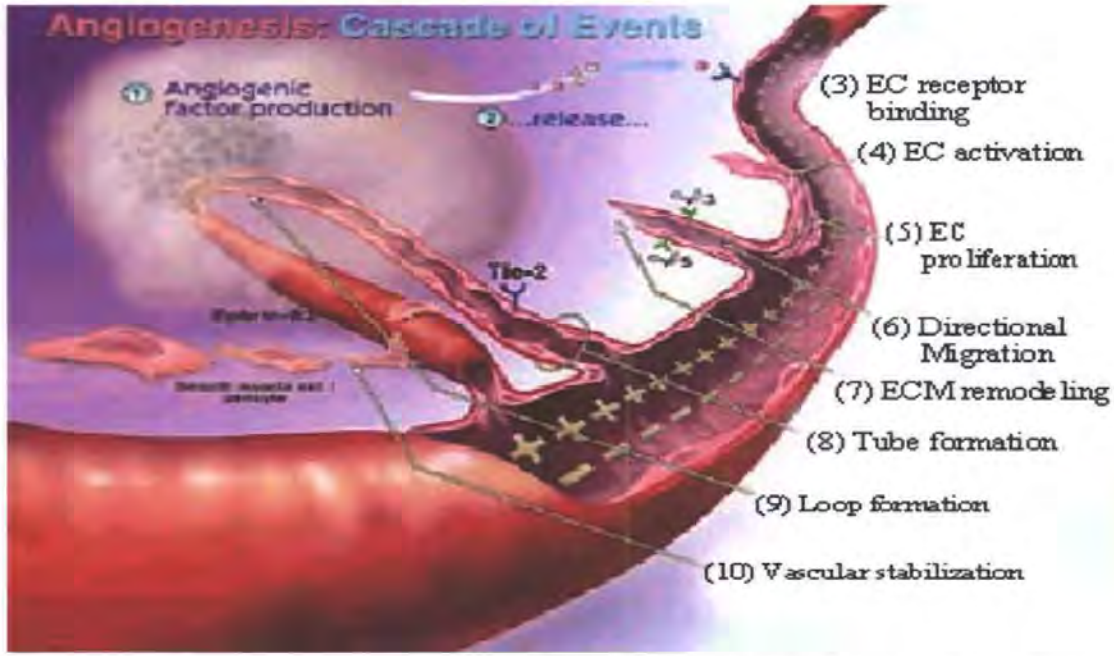
1.2 Angiogenesis

The term angiogenesis was coined in 1935 to describe the formation of new blood vessels in the placenta (Hertig, 1935). Angiogenesis is defined as the biological process by which new capillaries are formed from pre-existing vessels by sprouting i.e. cellular outgrowth (Battegay, 1995). It is different from vasculogenesis, which is the development of embryonic blood vessels (Risau, 1994).

In the adult male, angiogenesis only occurs in abnormal conditions such as wound healing, fractures, and in muscles during athletic training, while in the adult female it occurs normally in the menstrual period, and placenta formation (Weiss, 1991; Klagsbrun and D'Amore, 1991).

Although the molecular mechanism of the angiogenesis cascade is incompletely understood, the sequence of events leading to the formation of new vessels has been well documented. Studies of this process revealed that there are many steps involved including, degradation of the basement membrane (BM) of the pre-existing vessels by proteinases derived from activated microvascular endothelial cell (MVEC) (Gross *et al*, 1983; Stetler Stevenson, 1999), followed by the migration and proliferation of endothelial cells (ECs) from the degraded site towards the angiogenic stimulus (chemotactic factor) (Ausprunk and Folkman, 1977; Klein *et al.*, 1997). Then the lumen is formed (Folkman and Haudenschild, 1980), and individual sprouts join with each other to form loops, and lastly blood flow begins, pericytes appear and a new basement membrane is synthesized. Fig. 12 shows the process of angiogenesis.

Fig. 12. Process of angiogenesis: The process of angiogenesis occurs as an orderly series of events:



1. Diseased or injured tissues produce and release angiogenic growth factors (proteins) that diffuse into the nearby tissues
2. The angiogenic growth factors bind to specific receptors located on the endothelial cells (EC) of nearby preexisting blood vessels
3. Once growth factors bind to their receptors, the endothelial cells become activated. Signals are sent from the cell's surface to the nucleus. The endothelial cell's machinery begins to produce new molecules including enzymes
4. Enzymes dissolve tiny holes in the sheath-like covering (basement membrane) surrounding all existing blood vessels
5. The endothelial cells begin to divide (proliferate), and they migrate out through the dissolved holes of the existing vessel towards the diseased tissue (tumor)
6. Specialized molecules called adhesion molecules, or integrins (avb3, avb5) serve as grappling hooks to help pull the sprouting new blood vessel sprout forward
7. Additional enzymes (matrix metalloproteinases, or MMP) are produced to dissolve the tissue in front of the sprouting vessel tip in order to accommodate it. As the vessel extends, the tissue is remolded around the vessel
8. Sprouting endothelial cells roll up to form a blood vessel tube
9. Individual blood vessel tubes connect to form blood vessel loops that can circulate blood
10. Finally, newly formed blood vessel tubes are stabilized by specialized muscle cells (smooth muscle cells, pericytes) that provide structural support. Blood flow then begins

Adapted from the Angiogenesis Foundation site (http://www.angio.org)

The process of angiogenesis can be physiological or pathological (Table 8).

1.2.1 Physiological angiogenesis:

Physiological angiogenesis is an essential process to maintain a constant oxygen and nutrient supply to newly growing or recycling tissues (Thompson, 1989). This type of angiogenesis is a very tightly controlled process (Moses and Langer, 1991). It occurs normally in children particularly in osteogenesis in which the new blood vessels invade the previously avascular cartilage prior to the formation of bone (Weiss, 1991). It also occurs in wound healing, the process which cannot occur without angiogenesis. Angiogenesis plays more than a nutritive role, the endothelial cell is also an organizer and a regulator of healing (Arnold and West, 1991).

Physiological angiogenesis occurs as a normal process in the female reproductive organs (ovary and uterus), and in placenta at the time of pregnancy (Reynolds *et al*, 1992). Rapid growth and regression of female reproductive tissues are accompanied by equally rapid changes in rates of blood flow (Reynolds, 1986).

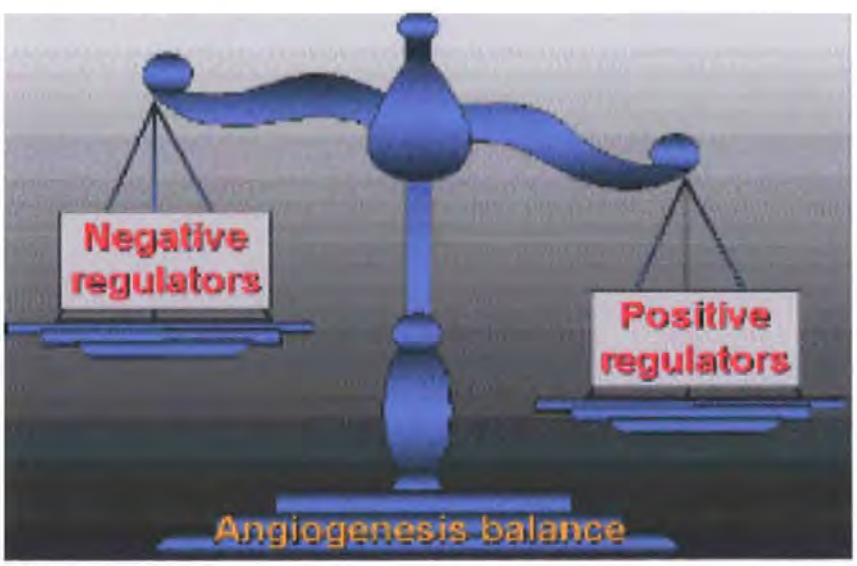
1.2.2 Pathological angiogenesis:

Pathological angiogenesis occurs in what are known as angiogenic diseases, in which the normal actions of factors involved are perturbed in the environment of a disease to ultimately cause pathological neovascularisation (Schultz and Grant, 1991). In other words under normal homeostatic conditions, most tissues express relatively high levels of angioinhibitory substances, maintaining the vasculature in a quiescent state (Bouck *et al.*, 1996). However, during disease states, such as neoplasia, the balance is shifted in favour of endothelial activation and induction of angiogenesis (Fig 13) (Hanahan *et al.*, 1996; Bouck *et al.*, 1996).

Table 8. Physiological and pathological angiogenesis:

Physiological	Pathological
Ovulation Development of the corpus luteum Embryogenesis Lactating breast Immune response Wound repair	**Neoplasia** Solid and haematological tumours **Cardiovascular disorders** Atherosclerosis Haemangiomatosis **Ocular disorders** Diabetic retinopathy Neovascular glaucoma Retrolental fibroplasia **Chronic inflammatory diseases** Diabetes Psoriasis Pyogenic granuloma Rheumatoid arthritis Systemic sclerosis

Fig. 13. Angiogenesis balance:



1.2.3 Angiogenic and anti-angiogenic factors

Angiogenesis is a finely regulated process. Many substances are now known to modulate angiogenesis i.e. pro- and anti-angiogenic factors (Table 9&10). Most of the angiogenic factors are non-specific for the angiogenesis process (i.e. non-specific endothelial cell mitogens or chemotactic or both), or indirect (i.e. do not induce angiogenesis by themselves, but by recruiting cells which produce direct angiogenic factors). Humoral angiogenic factors include polypeptides and non-peptide low molecular weight compounds. In addition, many anti-angiogenic factors have been identified.

In addition to the humoral angiogenic factors, many cells including endothelial cells, macrophages/monocytes (Polverini *et al.*, 1977), mast cells (Azizkhan *et al.*, 1980), pericytes (Orlidge and D'Amore, 1987), and tumour cells influence the formation of new blood vessels by secreting soluble angiogenic and antiangiogenic molecules.

Pathological angiogenesis (angiogenic disease) is probably the consequence of an imbalance between factors stimulating and factors inhibiting angiogenesis, or an inadequate expression of constituents in their signalling pathway (Schweigerer and Fotsis, 1992). In order to prevent the development of angiogenic diseases, at least one of the main steps of angiogenesis process must be stopped (i.e. basement membrane degradation, endothelial cell migration, proliferation, lumen formation, etc).

Complete inhibition of angiogenesis should be well tolerated in most adults since, under physiologic conditions, angiogenesis is required only for wound healing and reproduction.

However, a better understanding of angiogenesis process cascade of events will facilitate the development of anti-angiogenic factors.

Table 9. Examples of proangiogenic factors

Growth factors and cytokines	Peptides	Matrix-degrading enzymes	Steroid hormones	Lipids	Others
Basic and acidic fibroblast growth factors Vascular endothelial growth factor Placental growth factor Angiopoietins 1 and 2 Scatter factor Epidermal growth factor Transforming growth factor α and β. Interleukin 8 Granulocyte macrophage-colony-stimulating factor. Tumor necrosis factor α Angiogenin Thymidine phosphorylase Midkine Pleiotrophin Proliferin	Substance P Angiotensin II	Urokinase and tissue-taype plasminogen activator. Matrix metalloproteinases. Hyaluronidase.	Estrogen	Prostaglandins E1 and E2. Platelet activating factor. .	Nitric oxide Adenosine Histamine Copper

*Adopted and modified from Campbell et al., 1998.

Table 10. Examples of antiangiogenic substances

Endogenous substance	Encrypted molecules	Anticancer treatments	Antibiotics	Inhibitors of matrix tumourover	Others
Interferon α, βand γ Thrombospondin 1 and 2 Interleukins 1, 4 and 12 Interferon γ-inducible protein 10 Platelet factor 4. Tissue inhibitor of matrix metalloproteinase.	Angiostatin Endostatin Laminin Fibronectin Prolactin	Bleomycin Methotrexate Radiotherapy Antiestrogens (tamoxifen) Hyperthermia Retinoids	Linomide Minocycline Suramin TNP-470 and other fumagillin derivatives	Synthetic inhibitors of matrix metalloproteinases. Inhibitors of collagen synthesis.	Aspirin and cyclo-oxygenase-2 inhbitors. Captopril. Vitamin D3 analogues. Antibodies to αvβ3 and αvβ5 integrins.

*Adopted and modified from Campbell et al., 1998.

In all types of angiogenesis, either under physiologic or pathologic conditions, endothelial cell activation is the first process to take place. Cytokines from various sources are released in response to hypoxia or ischaemia. It is suggested that vascular endothelial growth factor (VEGF) is a major player in angiogenesis initiation based on its ability to induce vasodilatation via endothelial nitric oxide (NO) production and its endothelial cell permeability increasing effect (Ziche *et al.*, 1997). This allows plasma proteins to enter the tissue to form a fibrin-rich provisional network (Dvorak, 1986). The observation that VEGF production is under control of hypoxia inducible factor (HIF) strengthens the suggestion of an early involvement of VEGF in the angiogenic response. The level of HIF-1α mRNA itself does not change in response to hypoxia, however HIF-1α's oxygen dependent degradation via the ubiquitin-proteasome pathway is inhibited (Wenger *et al.*, 1997; Huang *et al.*, 1998). This degradation is usually stimulated by the von Hippel-Lindau (VHL) gene product p(VHL), the loss of which has been demonstrated as a characteristic finding in clear cell carcinoma of the kidney, and is the key genetic variant in the pathogenesis of the VHL syndrome. The interaction between HIF-1α and pVHL is regulated through hydroxylation of a specific proline residue at position 564 (Jaakkola *et al.*, 2001). Hypoxia causes a rise in HIF-1, and this is maximal at 0.5% oxygen tension in vitro (Jiang *et al.*, 1996). It has previously been demonstrated in a mouse embryo model, that loss of HIF-1α leads to reduced expression of the proangiogenic vascular endothelial growth factor (VEGF) in response to hypoxia, leading to mouse foetal death (Ryan *et al.*, 1998). Inhibition of HIF-1 via knockout of the HIF-1β subunit leads to reduced in vitro and vivo xenograft tumour growth and angiogenesis, or alternatively in utero murine death (Maxwell *et al.*, 1997; Maltepe *et al.*, 1997). In vitro study of several bladder cancer cell lines has demonstrated increased levels of HIF-1α protein (not mRNA) in hypoxia, with matched rises in VEGF induction. This upregulation of VEGF was lost at confluence in some,

though not all superficial cell lines, but not in invasive phenotype cells, in keeping with their more solid morphology in vivo (Jones *et al.*, 2001). Invasive cell lines also demonstrated increased levels of HIF-2α protein. Moreover, VEGF receptor (VEGFR) expression is up-regulated under hypoxic or ischaemia conditions (Forsythe *et al.*, 1996).

Besides the already mentioned proangiogenic factors, many others have now been identified in various settings of physiologic and pathologic angiogenesis. Among them are bFGF (Gospodarowicz, 1974), aFGF (Roberts *et al*, 1986), TGF-α and TGF-β, granulocyte macrophage-colony-stimulating factor, epidermal growth factor, interleukin-1 (IL-1), scatter factor, platelet-activating factor, IL-8 (Koch *et al*, 1992), and substance P (Bouck *et al.*, 1996; Yoshida *et al.*, 1997). Their effects can be either directly or indirectly on the endothelium via activation of surrounding cells to produce other factors with proangiogenic activity or modulation of receptors/receptor activities (Yoshida *et al.*, 1997; Giraudo *et al.*, 1998).

Hypoxia is an important environmental factor that leads to neovascularization. In the case of tumour growth, however, cancer-causing genetic changes, possibly in conjunction with environmental influences, are able to induce angiogenesis as well (Rak *et al.*, 1995; Okada *et al.*, 1998). Many oncogenes, among which *c-myb, sis,* and *src,* have been shown to stimulate the expression of a wide variety of molecules that induce angiogenesis. Furthermore, mutant *ras* oncogenes strongly up-regulated the proangiogenic factors TGF-α, TGF-β, and VEGF. Activated oncogenes can also indirectly contribute to the angiogenic phenotype by affecting the production and activation of BM and ECM-degrading enzymes (Bouck *et al.*, 1996; Okada *et al.*, 1998).

Tumour suppressor genes have now also been identified to play a role in angiogenic activities of cells. Inactivation of p53, for example, down-regulated the antiangiogenic ECM component thrombospondin (Dameron *et al.*, 1994; Grossfeld *et al.*, 1997). Besides the involvement of tumour cell-associated changes in p53, this tumour suppressor gene also plays a role in endothelial cell-mediated control of angiogenesis. Moreover, the multinucleated variant endothelial cells expressed a mutant p53 type, which may be indicative for loss of endothelial cell growth control (Satoh *et al.*, 1998). As with tumour cells, it is most likely that in vivo a combination of mutations in various tumour suppressor genes and oncogenes leads to a proliferative proangiogenic character of the endothelial cells.

1.2.4 Methods for studying angiogenesis:

The development of simple, reliable, reproducible, quantitative *in vivo* and *in vitro* assay procedures is essential to enable the investigator to test both angiogenesis inducers and angiogenesis inhibitors.

Several methods have been developed to test these agents. These methods have many drawbacks including that they are difficult to perform, extremely time consuming, expensive, only qualitative, require large amounts of expensive test material, and they give variable results (Table 11).

Table 11. Models of angiogenesis

In vitro	In vivo
Endothelial cell culture system (Chemotaxis and proliferation). Human placental blood vessels fragments in fibrin gel. Rat aorta explants.	Corneal micropocket model. Chick embryo chorioallantoic membrane assay. Rat subcutaneous air sac model. Angiogenesis in vivo using basement membrane extracts (Matrigel).

1.2.5 Tumour angiogenesis

The idea that solid tumour growth is dependent on the induction of neovascularization originated in the 1960s from experiments in which tumours were grown in isolated perfused organs (Folkman, *et al.*, 1963. Folkman, *et al.*, 1966). In 1971, Folkman and co-workers showed that a soluble extract from solid tumours was able to induce microvascular growth in the absence of tumour itself (Folkman, *et al.*, 1971). Subsequently, Folkman put forward the hypothesis that "Once tumour take has occurred, every further increase in tumour cell population must be preceded by an increase in new capillaries which converge upon the tumour." Many tumours in humans persist in situ for months to years without neovascularization but then become vascularized when a subgroup of cells in the tumour "switches" to an angiogenic phenotype. Tumours can accomplish the switch from an antiangiogenic to an angiogenic phenotype by either upregulating the expression of angiogenesis inducers or downregulating inhibitors, and in some instances, both mechanisms are operative (Hanahan *et al.*, 1996; Volpert *et al.*, 1997; Campbell *et al.*, 1998). This switch is usually accompanied by rapid growth of the tumour.

Stimuli, which may promote this angiogenic switch, include metabolic stresses, such as hypoxia, acidosis and hypoglycaemia, as well as mechanical strain due to tissue growth, and inflammatory or immune cell activation. Genetic mutation, causing dysregulation of factors involved in angiogenesis, may affect the balance of angiogenic factors directly.

It is the switch to the angiogenic phenotype that may allow clinically dormant microscopic neoplastic disease to grow and become clinically apparent, and there is extensive experimental evidence to support the claim that tumours and metastases are angiogenesis dependent, (Folkman, 1990). Folkman first described the need for tumours to develop their own blood supply to grow beyond 2–3mm^3 in 1971 (Folkman, 1971).

The onset of angiogenic activity enables rapid expansion of the tumour population and increases the risk of metastasis, and this can be stimulated by inflammation.

The process of tumour angiogenesis begins with the enzymatic degradation of the basement membrane of a postcapillary venule and the breakdown of the interstitial matrix. This permits chemotaxis of endothelial cells towards the angiogenic stimulus and proliferation into the surrounding stroma of the existing endothelium. Three distinct zones of angiogenesis result in: a migratory zone, a proliferative zone and a zone of maturation, where functional vessels can be identified (Polverini, 1995). Capillary endothelial cells become organized into tubular structures and capillary sprouts form anastomoses between themselves and components of the host vasculature, following which initial blood flow can be seen (Baillie *el at*, 1995; Hayes, 1994; Ferrara, 1995). These new vessels migrate towards the tumour to form the basis of the tumour stroma, together with interstitial fluid, interstitial collagens, fibrin, fibronectin, vitronectin, glycosaminoglycans, proteoglycans, fibroblasts and some inflammatory cells (Senger, *et al.*, 1995; Dvorak, *et al.*, 1995a).

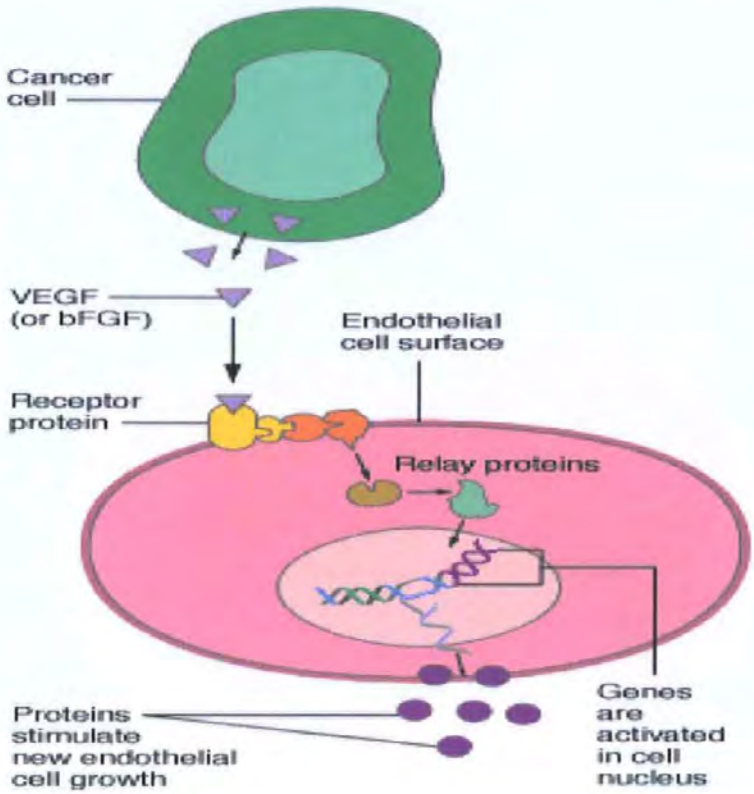
There are many important differences between the structure of normal and tumour vasculature. Vessels which grow in response to a tumour stimulus are mainly composed of endothelial cells, whilst normal capillaries usually also contain vascular pericytes. The tumour vasculature appears as a disorganised network that consists of many abnormal blood vessels including sinusoidal vessels, giant capillaries and blood channels with a discontinuous endothelial lining. Although normal vessels may also be recruited from the host tissues (Blood & Zetter, 1990; Baillie *et al.*, 1995). As the tumour vasculature lacks innervation and has low effective capillary density, this leads to hypoxia and acidosis within the tumour stroma. This is further aggravated by the fibrinous and later a collagenous matrix that separates tumour cells from blood vessels. Tumour vasculature also has leaky blood vessels and poor lymphatic drainage, which

causes raised interstitial hydrostatic pressure within the tumour core. This can lead to occlusion of the tumour interior blood vessels with consequent ischaemia and eventually necrosis within the tumour core. Failure of the tumour vasculature can be precipitated by hypotension, alterations in blood coagulability or viscosity, or by damage to endothelial cells resulting in coagulation and the blocking of nutrient vessels with white cells (Blood & Zetter, 1990; Battegay, 1995; Baillie, *et al,* 1995; Dvorak, *et al.*, 1986).

As previously mentioned, in the prevascular phase, the tumour is rarely larger than 2 to 3 mm^3 and may contain a million or more cells (Folkman, 1995). Such asymptomatic lesions are sometimes directly visible on the skin or cervix or in the bladder, but usually they are clinically undetectable. Cells in prevascular tumours or dormant micrometastases may replicate as rapidly as those in expanding, vascularized tumours, but without the growth of new vessels the rate of proliferation of such cells reaches equilibrium with their rate of death (Idem, 1995; Holmgren, *et al.*, 1995). A tumour consists of 2 interdependent compartments: the parenchyma (tumour cells) and the stroma (supporting cells and connective tissue). A blood vessel network is contained within the stroma, which provides nutrients, gas exchange and waste disposal, and allows the cancer cells to establish continuity with the normal vasculature (Dvorak, 1986; Ferrara, 1995; Gasparini & Harris, 1995). In addition, the stroma may act as a barrier, blocking recognition of the tumour cells by inflammatory and immune cells (Dvorak, 1986; Blood & Zetter 1990. Folkman, 1995b).

What actually causes the tumour angiogenic switch is currently under investigation, however it may occur due to the down-regulation of an inhibitor of angiogenesis under the control of a tumour suppressor gene such as p53, aided by the expression of angiogenic factors such as basic fibroblast growth factor (bFGF) or vascular endothelial growth factor (VEGF) (Samoto *et al.*, 1993; Campbell *et al.*, 1998) (Fig. 14).

Fig. 14. The angiogenesis-signaling cascade:

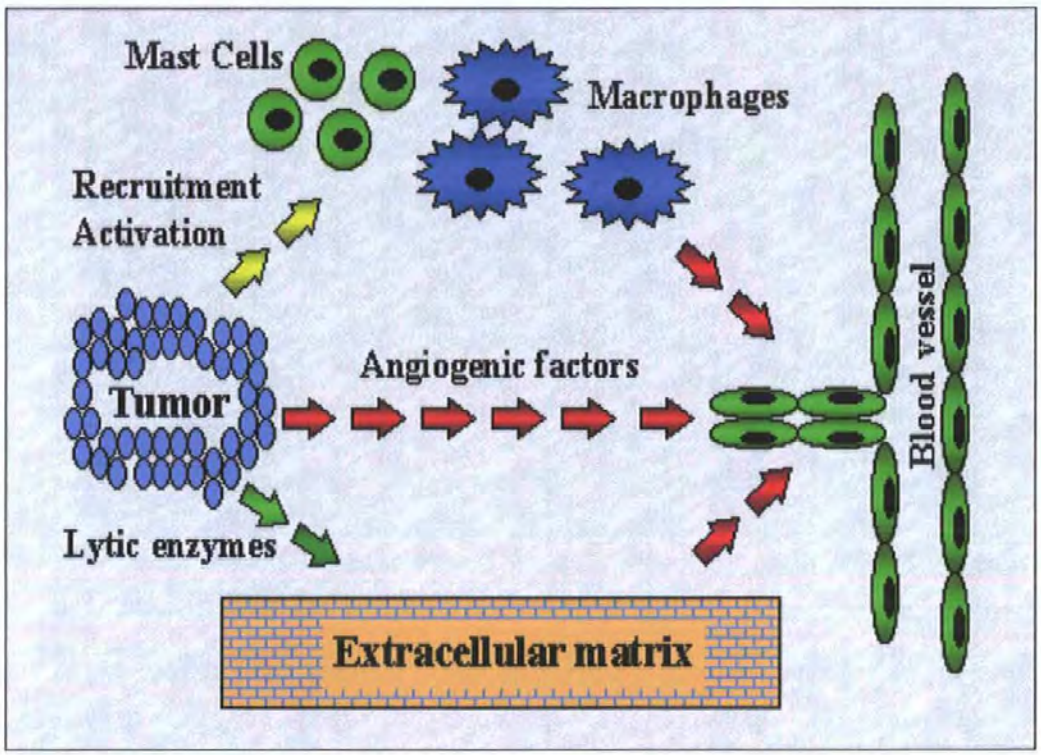


VEGF and bFGF are first synthesized inside tumour cells and then secreted into the surrounding tissue. When they encounter EC, they bind to specific protein (receptors), sitting on the outer surface of the cells. The binding of either VEGF or bFGF to its appropriate receptor activates a series of relay proteins that transmits a signal into the nucleus of the EC. The nuclear signal ultimately prompts a group of genes to make products needed for new endothelial cell growth.

There is no doubt that tumour angiogenesis is the result of the concerted interplay of a multitude of different cytokines, growth factors and cell adhesion molecules. Some of these interact directly with the cells responsible for neovascularisation, such as endothelial cells and pericytes, and others stimulate cells such as macrophages, mast cells and fibroblasts to produce primary or secondary angiogenic factors (Hayes, 1994; Miles, 1999) (Fig. 15).

Angiogenesis may not always appear as the initial step in tumour formation. In cervical neoplasia, angiogenesis typically occurs prior to the gross appearance of tumour: in breast and bladder, a marked increase in angiogenesis occurs coincidentally with tumour development; and in melanoma and ovarian carcinoma, angiogenesis occurs after tumour formation (Folkman, 1990). Experimental and clinical data indicate that most tumours occur without angiogenic activity, exist in the *in situ* stage without neovascularization for a long period of time, and then become vascularized when a subset of cells within the tumour switches to the angiogenic phenotype (Folkman, 1995).

Fig. 15. Source of angiogenesis factors: Not only cancer cells but also inflammatory cells that infiltrate the tumour and the extracellular matrix can be a source of angiogenesis factors.



1.2.6 Angiogenesis and Metastasis

Experimental and clinical evidence suggests that the process of metastasis is also angiogenesis dependent. For a tumour cell to metastasize successfully, it must breach several barriers and be able to respond to specific growth factors (Nicolson, 1988; Watanabe *et al.*, 1991; Zetter, 1998). Thus, tumour cells must gain access to the vasculature in the primary tumour, survive the circulation, arrest in the microvasculature of the target organ exit from this vasculature, grow in the target organ, and induce angiogenesis (Weidner *et al.*, 1991; Weinstat-Saslow *et al.*, 1994). Therefore, angiogenesis appears to be necessary at the beginning as well as at the completion of the metastatic cascade.

In experimental animals, tumour cells are rarely shed into the circulation before a primary tumour is vascularized, but they can appear in the circulation continuously after neovascularisation (Liotta, *et al.*, 1980). The number of cells shed from the primary tumour correlates with the density of tumour blood vessels as well as with the number of lung metastases observed later. Tumour cells can enter the circulation by penetrating through proliferating capillaries that have fragmented basement membranes and are leaky (Dvorak *et al.*, 1999). Further, angiogenic factors from tumours such as bFGF and VEGF induce increased production of plasminogen activator and collagenases in proliferating endothelial cells, thus further contributing to degradation of basement membranes. These degradative enzymes may facilitate the entry of tumour cells into the circulation. Tumour cells that have successfully metastasized may not immediately become neovascularized after reaching the target organ. Such a metastasis lacking angiogenic activity for any of a variety of reasons may remain as a microscopic tumour of 100 to 200 μm diameter indefinitely (Chambers, 1999; Folkman, 1995). It is generally assumed that in human dormant micrometastases (e.g., metastases appearing

5–10 years after removal of a breast cancer), tumour cells are not cycling or are in G0. Increasing experimental evidence, however, indicates that micrometastases can be held dormant by blocked angiogenesis that results in a balance of tumour cell proliferation and apoptosis (Holmgren *et al.*, 1995; Folkman, 1995). Finally, experimental metastases are as susceptible as primary tumours to control by specific angiogenesis inhibitors (O'Reilly *et al.*, 1996; Teicher *et al.*, 1992). For these inhibitors, the microvascular endothelial cell is the only target; tumour cells are not directly affected in vitro.

In many types of human cancer including bladder cancer, microvessel density in a histologic section of the tumour is an independent prognostic indicator of the risk of future metastases (Weidner *et al.*, 1991; Campbell *et al.*, 1998). Since the report of Weidner and colleagues in 1991, there have been many different reports that in human breast cancer there is a positive association between tumour angiogenesis and metastatic risk (Folkman, 1998).

Because of the clonal origin of metastases (Kerbel *et al.*, 1988; Weinstat-Saslow *et al.*, 1994), a primary tumour containing a high proportion of angiogenic malignant cells is more likely to generate metastases that are already angiogenic when they arrive at the target tissue. In contrast, there are a number of reports of no association between tumour angiogenesis and metastatic risk (Folkman, 1998; Gasparini and Harris, 1999).

1.2.7 Angiogenesis in bladder cancer

The initial observations demonstrating that bladder cancer is partly an angiogenic disease were provided by Chodak and colleagues, who showed that bladder tumour fragments could induce neovascularity when implanted in the cornea of rabbits, whereas normal bladder tissue could not (Chodak *et al.*, 1980; Chodak *et al.*, 1997). Subsequent studies demonstrated that urine from bladder cancer patients is also strongly angiogenic, suggesting secretion of angiogenic factors by these tumours (Chodak *et al.*, 1988; Chodak *et al.*, 1981). Chodak's group eventually isolated an angiogenic substance from the urine of mice with bladder cancer and showed that it was a heparin-binding growth factor with properties similar to those of the FGF family of molecules (Chodak *et al.*, 1988). A long list of agents involved now exist, and only those which have been described in particular reference to bladder cancer are listed in Table 12.

Multiple lines of evidence have now converged to demonstrate that bladder tumours are dependent on angiogenesis for progression, growth, and metastasis. Fluorescein angiography reveals markedly increased uptake in papillary tumours and carcinoma in situ compared with normal urothelium, indicating that neovascularity is acquired relatively early during bladder tumourigenesis (Zimmern *et al.*, 1995).

Published studies describing the association between bladder cancer and angiogenesis are shown in Table 13. Data with regard to superficial bladder cancer remains controversial, possibly due to difficulties in quantification within a friable papillary tumour (Dinney *et al.*, 1998; Ozer *et al.*, 1999). Technical problems, such has unrepresentative sampling of microvessel hotspots, where other investigators quote mean MVD, and the differing nature of tissue, either transurethral resection versus cystectomy specimens may explain some inconsistencies between series.

Table 12. Pro- and anti-angiogenic factors described in bladder cancer.

Pro-angiogenic factors	Anti-angiogenic factors
Basic fibroblast growth factor (bFGF) (Gazzaniga et al., 1999).	Cortisone (Lee et al., 1990)
Acidic fibroblast growth factor (aFGF) (Chopin et al., 1993)	Thrombospondin1(TSP-1) (Grossfeld et al., 1997)
Vascular endothelial growth factor (VEGF) (Crew et al., 1999)	Angiostatin (Beecken et al., 2001)
Epidermal growth factor (EGF) (Perrotte et al., 1999)	Interferon-α (Slaton et al., 1999)
Thymidine phosphorylase (TP) (Arima et al., 2000)	
Scatter factor/hepatocyte growth factor (SF) (Tamatani et al., 1999)	
Midkine (MK) (O'Brien et al., 1996)	
Hyaluronic acid (HA) (Lokeshwar et al., 1997)	
Angiogenin (Miyake et al., 1999)	
Cyclooxygenase 2 (Cox 2) (Yoshimura et al., 2001)	
Hypoxia inducible factors 1&2 (HIF-1/HIF-2) (Talks et al., 2000)	
EIF-4e (Crew et al., 2000)	
Matrix metalloproteinases. (Papathoma et al., 2000)	
Urokinase plasminogen activator (Hasui et al., 1996)	
Mutant p53 (Reiher et al., 2001)	
Interleukin-8 (Inoue et al., 2000)	

The published literature regarding invasive tumours is more convincing (Streeter *et al.*, 2002).

Quantitative immunohistochemistry analysis of endothelial antigens such as Factor VIII-related antigen, CD31, and CD34 also supports the angiogenic dependence of bladder cancer. Microvessel density has been demonstrated to be a useful prognostic indicator in a variety of malignancies, including melanoma (Srivastava *et al.*, 1988; Barnhill *et al.*, 1992), breast cancer (Weidner *et al.*, 1992; Horak *et al.*, 1992) and prostate cancer (Weidner *et al.*, 1993; Brawer *et al.*, 1994). In general, increased microvessel density counts have been associated with tumour progression and decreased overall survival (Srivastava *et al.*, 1988; Barnhill *et al.*, 1992; Weidner *et al.*, 1992; Horak *et al.*, 1992; Weidner *et al.*, 1993; Brawer *et al.*, 1994). The relationship between microvessel density count and tumour progression has been examined in patients with bladder cancer (Bochner *et al.*, 1995; Jaeger *et al.*, 1995; Dickinson *et al.*, 1994; O'Brien *et al.*, 1995; Campbell *et al.*, 1997). Jaeger *et al.*, demonstrated a significant correlation between tumour angiogenesis (as determined by microvessel density) and lymph node metastases in 41 patients with invasive bladder cancer (Jaeger *et al.*, 1995). Dickinson *et al.*, showed microvessel density to be an independent prognostic indicator of disease progression in 45 patients with invasive bladder cancer followed for a median of 37 months (Dickinson *et al.*, 1994). The most comprehensive such study was by Bochner and colleagues, in which 164 patients with invasive disease managed with radical cystectomy were retrospectively divided into groups with low, intermediate, and high microvessel density. The 5-year recurrence-free rates for these three groups were 81%, 44%, and 32%, respectively, and the overall 5-year survival rates were 68%, 44%, and 34%, respectively (Bochner *et al.*, 1995).

Table 13. Studies of microvessel density (MVD) and prognosis in bladder cancer:

Study	No	Histology	Stained for	p-value (rec. free surv)	p-value (overall surv)	Uni/ multivariate	Comments
Dickinson (1994)	45	Invasive (TURBT)	CD31	N/A	0.026	Univariate	MVD not associated with stage and grade, but high →MVD-2.5x↑mortality.
Bochner (1995)	164	Invasive (Cystectomy)	CD34	<0.0001	0.0007	Multivariate	↑ MVD correlates with stage at presentation, recurrence and survival
Jaeger (1995)	41	Invasive (Cystectomy)	Factor VIII	N/A	N/A	N/A	↑MVD correlates with lymph node metastases at presentation
Philp (1996)	113	Superficial or invasive (TURBT)	CD31	N/A	0.01	Multivariate	MVD correlated with stage at presentation
Grossfeld (1997)	163	Invasive (Cystectomy)	CD34	N/A	N/A	N/A	↑ MVD associated with ↓survival and low Thrombospondin-1
Bochner (1997)	161	Invasive (Cystectomy)	CD34	<0.001	0.003	Multivariate	↑MVD associated with progression, and loosely with↑p53
Dinney (1998)	54	T1 (TURBT& Cystectomy)	Factor VIII	N/A	N/A	Univariate	No association between MVD and recurrence or progression.
Hawke (1998)	42	Invasive (Cystectomy)	Factor VIII	N/A	0.04	Univariate	MVD of no independent prognostic value.
Chaudhary (1999)	88	Invasive (Cystectomy)	CD31	0.03	0.02	Univariate	↑ MVD associated with nodal metastases at cystectomy,↑recurrence, ↓survival.
Ozer (1999)	20	G3 T1 (TURBT)	Factor VIII	0.002	0.01	Univariate	↑ MVD associated with ↑ recurrence and ↓survival.
Inoue (2000)	51	Invasive (Cystectomy)	CD34	0.048	N/A	Multivariate	↑ MVD predicts recurrence in patients given neoadjuvant MVAC chemotherapy
Sagol (2001)	80	Superficial (TURBT)	CD31	N/A	N/A	Univariate	MVD did not differ from Ta to T1, or predict recurrence. ↑MVD associated with ↑grade.

TURBT=transurethral resection of bladder tumour; Invasive=stage T2 or greater; N/A=data not available.

* Adopted from Streeter et al., 2002.

On multivariate analysis, microvessel density proved to be an independent prognostic indicator when evaluated in the presence of histological grade, pathological stage, regional lymph node status, and p53 status (Bochner *et al.*, 1995; Bochner *et al.*, 1997). High microvessel density was also associated with a twofold to threefold higher risk of death from invasive bladder cancer in other recent studies (Hawke *et al.*, 1998; Dickinson *et al.*, 1994). However, Chopin and colleagues found no correlation between microvessel density and relapse-free survival in 49 patients with negative lymph nodes who had been treated with radical cystectomy (Chopin *et al.*, 1996). Differences in patient populations, statistical power, and immunohistochemistry protocols could account for such disparate findings.

A further recent series of 51 cystectomy cases using hot spot analysis showed high MVD predict recurrence in patients given neoadjuvant MVAC chemotherapy (Inoue *et al.*, 2000).

Assays for the expression of a variety of angiogenic factors may also have predictive power for bladder cancer. Increased expression of thymidine phosphorylase has correlated with tumour grade and stage (O'Brien *et al.*, 1997; O'Brien *et al.*, 1996) and midkine (a member of a family of heparin-binding growth factors) with poor outcomes in patients with invasive disease (O'Brien *et al.*, 1996). Urinary levels of VEGF (Crew *et al.*, 1997; Crew *et al.*, 1998), aFGF (acidic fibroblast growth factor) (Chopin *et al.*, 1993), bFGF (basic fibroblast growth factor) (Nguyen *et al.*, 1993; Bochner *et al.*, 1997), and scatter factor (Rosen *et al.*, 1997) have all correlated with disease status and, in many cases, with tumour grade and stage. Urinary bFGF levels have been evaluated most extensively. Bochner *et al.*, in 1997 reported a correlation between urinary bFGF and microvessel density, again indicating a functional role for this growth factor in the induction of angiogenesis in this disease. In the study by Nguyen and colleagues, urinary levels of bFGF were found to have better predictive value than cytological

analysis for the diagnosis of recurrent bladder cancer (Nguyen *et al.*, 1993). More recent investigation in a series of 185 resections showed no correlation between VEGF protein, recurrence or survival (Chow *et al.*, 1999), with a separate report correlating VEGF with increasing grade of tumour, but not with MVD (Ogura *et al.*, 1998).

Bernardini *et al.*, in 2001 showed in his study a significant difference in serum vascular endothelial growth factor in healthy controls and patients with bladder cancer. The serum level was significantly associated with tumour stage, grade, vascular invasion and carcinoma in situ. Patients with metastasis had significantly higher levels than those with localized diseases (Bernardini *et al.*, 2001).

1.3. Radiotherapy

Radiotherapy treatments aim to deliver a homogeneous dose of irradiation to an accurately localized tumour volume and to minimize the effect on the surrounding normal tissue. This requires the target volume to be defined as accurately as possible from clinical examination, radiological investigations, which frequently includes CT scans, and from knowledge of the natural history and patterns of spread of any particular type of malignancy.

Radiation therapy is one of four approaches to the treatment of cancer. The other three are surgery, chemotherapy, and biological therapy. Since each type of cancer is unique, research has helped physicians determine which treatment or combination of treatments is appropriate for each type of cancer. Radiation therapy may be used alone or in combination with surgery, chemotherapy, and/or biological therapy. Radiation therapy may be used in an attempt to cure the cancer or to treat unpleasant symptoms the cancer is causing such as pain or bleeding. The most common types of cancer that radiation therapy is used for are brain tumours, head and neck cancers, lung cancer, breast cancer, prostate cancer, skin cancer, rectal cancer, cervix and uterine cancers, lymphoma, and sarcoma (Robert *et al.*, 2000).

Radiation therapy for the treatment of cancer may be given in two different ways. External beam irradiation is the most common method. The radiation is delivered to a specific area of the body using a large machine similar to an x-ray machine. The treatment is given each day, Monday through Friday, for 1 to 8 weeks depending on the type of cancer and the reason for the treatment. The treatment may be given once a day, twice a day, or as many as three times a day for certain cancers.

The other method of delivering radiation treatment is called brachytherapy. In this method, a source of radiation in the shape of needles or seeds is implanted in the body. This treatment is often given before or after external beam irradiation as a way of

increasing the radiation dose to only the tumour. Brachytherapy is often used in cervix, uterine, and prostate cancers, some head and neck cancers, and sarcomas. Some of the implants stay in place permanently, whereas others are removed after 2 or 3 days.

The role of radiotherapy for a particular tumour type is determined partly by the average radiosensitivity of the tumour relative to adjacent normal tissues and partly by the probability that the tumour is localized to the irradiation field. Irradiation with photons (x-rays or gamma rays), electrons or high energy particles such as neutrons interact indirectly or directly with tissue to produce short lived ion radicals (Dizdaroglu *et al.*, 1992; Ward, 1994). These damage nuclear DNA leading eventually to programmed cell death (apoptosis), also called interphase death (Bryant, 1997). Cells undergoing apoptosis as an immediate consequence of radiation damage usually die in interphase within a few hours of irradiation, irrespective of and without intervening mitosis. They share distinct morphologic changes, including loss of normal nuclear structure and degradation of DNA that can be demonstrated by a classical pattern of "laddering" on DNA blots (Lowe *et al.*, 1993). Cells whose mitotic cycle time is short will show signs of radiation damage more quickly than those whose cycle time is long. However, it has been suggested by some authors that the level of DNA damage induced by radiation may be the primary determinant of radiosensitivity (Radford, 1986).

The other mechanism for cell killing is radiation-induced reproductive failure. Radiation in sufficient doses can inhibit mitosis; that is, the cell's ability to divide and proliferate indefinitely. The inhibition of cellular proliferation is the mechanism by which radiation kills most cells (Robert *et al.*, 2000).

The extent of DNA damage following radiation exposure depends on several factors. One of which is the level cellular oxygen (Littbrand *et al.*, 1969). Hypoxic cells are considered less radiosensitive compared to the well-oxygenated cells. In fact, anoxic cells require 2 to 3 times the radiation dose to produce an equivalent amount of cell kill,

as do oxygenated cells (Chapman *et al.*, 1999). Earlier reports have shown that severely anaemic patients undergoing radiotherapy were associated with poorer outcome. This was in contrast to the improvements seen in the outcome of radiotherapy patients who had received hyberbaric oxygen (Henk, 1981). Oxygen is believed to prolong the lifetime of the short-lived free radicals produced by the interaction of x-rays and cellular H_2O.

Ionizing radiation is consequently less efficacious in tumours with significant areas of hypoxia and necrosis. In contrast, damage following exposure to ionizing radiation is independent of cellular oxygen levels (Boice *et al.*, 1996).

The extent of DNA damage following radiation exposure is also dependent on the phase of the cell cycle (Elkind, 1997). Radiosensitivity varies both between and within the various phases. In general, the most sensitive phases are G2- and M-phases; the least sensitive are G1- and late S-phases (Bryant, 1997; Olive *et al.*, 1997), depending on the cell line studied. Irradiation of an asynchronously dividing population of tumour cells results in killing of a greater proportion of cells in G2- and M-phases, while surviving G1a- and S-phase cells may progress to more sensitive phases, a phenomenon known as reassortment. In contrast to indirectly ionizing radiation, DNA damage following exposure to directly ionizing radiation is less dependent upon the phase of the cell cycle (Raju *et al.*, 1978). Similarly, normal tissues show varying sensitivity both to the degree and timing of radiation damage.

Total dose of irradiation on tumours as well as normal tissues could be dramatically modified by dividing it into smaller parts or fractions. Empirically fractionated treatment regimes were developed which allowed for higher total doses to be delivered obtaining better local tumour control but without a high incidence of normal tissue damage. Fractionated treatment spares normal tissue because sublethal damage is repaired between daily fractions of treatment and normal tissues are allowed to

repopulate if the overall treatment time is sufficiently long. Many tumours have a relatively poor blood supply and contain hypoxic regions, which are relatively resistant to radiotherapy. Increasing the treatment time using a fractionated regimen allows surviving hypoxic tumour cells to re-oxygenate and additionally cells are allowed to re-assort into the more radiosensitive phases of the cell cycle (Abeloff *et al.*, 2000).

Generally acute reactions occur within 3 months of treatment, late reactions occur at more protracted intervals. Tissues having a high proliferative rate, such as skin and mucosal surfaces, express damage more rapidly (acute reacting), whereas slowly dividing tissue are late reacting. In particular it is possible that damage to vascular endothelial cells is responsible for many of the late radiation effects apparent clinically (Hopewell *et al.*, 1989).

1.3.1. Radiotherapy in bladder cancer

In the United States, the standard treatment for muscle invading TCC is radical cystectomy, while in Europe; external beam irradiation is a more common treatment (Kanady *et al.*, 1997). It is also recommended for patients deemed "unfit" for cystectomy, on the basis of either co morbid conditions or disease extent.

The goal of treatment with radiotherapy is definitive local (or loco-regional) control as an absolute prerequisite for cure. The correlation between local control and metastasis indicates that better systemic treatments, although badly needed, must not be developed without care for local control because local failure can be the source of further metastasis in patients initially free of distant disease (Pollack *et al.*, 1994). In a series of 379 patients treated at the Netherland Cancer Institute, 136 experienced a local recurrence and 120 of those recurred locally before regional or distant metastasis were detected (Moonen *et al.*, 1998).

Several authors, covering a large span of practices with various techniques and treatment schedules, have reported results of external radiotherapy. There are two fundamental approaches: the British-inspired schedule of 2.5Gy per fraction, five fractions per week for 4 weeks and the classical, continental schedule of 1.8 to 2 Gy per fraction, five fractions per week for 6 to 7 weeks. Since these approaches have never been tested against each other in a randomized trial, it is difficult to recommended one as being superior to the other. Data from larger series, summarized in table 14, show variation in local control and survival among the various series.

Overall, long-term local control and cure can be achieved in 60 to 80 per cent of clinical T1, 30 to 50 per cent of T2, and 20 to 40 per cent of T3 tumours with doses in the range 50 to 70 Gy (in 4 to 7 weeks). A dose-effect relationship has been shown to exist in transitional bladder cancer supporting the use of high-dose radiotherapy, either from radical radiotherapy series or from preoperative series suggesting a better down-staging with higher radiation doses (Moonen *et al.*, 1998; Nasslund *et al.*, 1994).

The patient presenting with a transitional cell bladder cancer is usually elderly. This important fact has often resulted in a recruitment selection between younger patients, who are frequently treated by radical surgery and older patients, in poorer general condition, who are more often referred to the radiation oncologist. Experience indicates that the success of radiotherapy may be favourably influenced by certain prognostic factors such as the absence of ureteral obstruction, the achievement of a complete transurethral resection before irradiation, the presence of papillary rather than a sessile tumour, and the clinical stage (Shipley *et al.*, 1985; Petrovich *et al.*, 2001).

It is of interest to note the importance of ureteral obstruction on survival in patients with T2 and T3 tumours. The 5-year survival of radiotherapy treated patients without ureteral obstruction was 54% versus 22% for those with ureteral obstruction (p=0.02) (Shipley *et al.*, 1985).

Table 14. Five-year local control and overall survival in selected larger series after definitive radiotherapy for bladder cancer.

Author	Stage	N	Dose	Local control (% at 5 years)	Overall survival (% at 5 years)
Blandy et al (1980)	T2-T3	352	50-55 Gy/20 fx	-	34
Goodman et al (1981)	T2-T3	470	50 Gy/20 fx	56	38
Yu et al (1985)	T1-T4	356	60-66 Gy/30-35 fx	20	38
Jenkins et al (1988)	T2-T3	182	50-55 Gy/20 fx	-	40
Davidson et al (1990)	T1-T4	709	50-64 Gy/ 20-30 fx	-	25
Jahnson et al (1991)	T1-T4	319	54-77 Gy/22-45 fx	51	18
Pollack et al (1994)	T2-T4	135	60-70 Gy/30-35 fx	32	27
Sengelov et al (1997)	T1-T4	94	58-63 Gy	-	7
Moonen et al (1998)	T2-T3	379	50-75 Gy/ 25-37 fx	40.3	22.2
Hayter et al (1999)	T2-T4	1,372	60 Gy	-	28

Fx = fractions, N = number of patients.

A study of 347 T3 (TCC) patients was reported from Western General Hospital of Edinburgh, Scotland. Patients were scheduled to receive 55 Gy in 20 fractions over a period of 18 days. Local tumour regression was assessed cystoscopically 6 months following completion of radiotherapy in 272 (82%) patients. Of these 272 patients, 112 (41%) showed complete tumour regression. The incidence of tumour regression was similar for those with ulcerative and solid tumours (about 52%), while it was 27% for papillary and 31% for mixed lesions, (p=0.024). Tumour grade and size (<5 cm vs. >5 cm) were also important factors influencing tumour regression (p=0.003). Grade I tumours had a regression rate of 17%, grade II of 31%, and grade III of 56% ($p < 0.0001$). The 5-year incidence of local tumour control was 10% for grade I, 14% for grade II, and 35% for grade III patients.

In multivariate analysis only patients age (p=0.06) and haemoglobin level (p=0.025) were important prognostic factors. Local tumour regression following radiotherapy was an important factor predicting survival. The 5- and 10-year survival of patients with complete tumour regression was 45% and 25%, respectively, as compared with 21% and 15%, respectively, for those with partial tumour regression (Quilty *et al.*, 1986).

In patients with muscle invasive bladder cancer it is difficult to compare the outcomes of definitive radiotherapy and surgical treatments. This is primarily due to the obvious selection bias-favouring patients who are to undergo radical cystectomy (Petrovich *et al.*, 2001). A review of 10 selected studies with a total of 4,368 patients treated with radiotherapy alone and using various treatment techniques, shows 5-year survival rates ranging from low of 7% to a high of 40% (Table 14).

In most series, despite negative selection, results are inferior to those observed with radical surgery. This is partly a result of the difficulty of rendering the bladder tumour free by external beam radiation alone, and the continued risk for developing new tumours in the organ left in situ. In most cases, treatments are delivered in five daily

fractions a week, ranging from 2.0 to 2.5 Gy to a total treatment dose of 55 to 65 Gy, with no interruptions. In designing the radiation fields to treat a patient with bladder cancer, the target is best defined by information from the planning cystogram, the CT scan, the cystoscopic examination, the bimanual examination, and having the bladder empty to assist set-up reproducibility and adequate coverage of the tumour at each treatment (Turner, *et al.*, 1994; Marks, *et al.*, 1992; Sur, *et al.*, 1993).

The bladder has a good tolerance to radiation; a diseased bladder may be more liable to side effects, either acute (frequent micturition) or late (haematuria, contracted bladder). Therefore, care must be taken to protect nontumoural areas of the bladder during the boost to 66 Gy. The rectosigmoid, small bowel, pelvic bones, and femoral heads are also critical tissues, which must not be injured by radiotherapy. The dose to the anterior rectal wall should be limited to less than 45 Gy. However, those dose limits are only guidelines and must be adapted according to each individual clinical situation. The planned schedule of 2 Gy per fraction, five fractions per week, must be accurately followed, in particular without gaps in the treatment, which could lengthen the total irradiation time. Indeed, tumours repopulate during radiotherapy and 6-7 weeks of irradiation represents a delicate trade-off between tumour sterilization and normal tissue sparing. Any gap in the treatment will break the equilibrium in favour of tumour repopulation. For example, in the radiotherapy series of Symonds *et al.*, 1990, a 2-3 week split in the treatment significantly decreased the 5 year survival from 35 to 22 per cent. Judgements as to the most appropriate treatment for an individual require a detailed clinical and radiological assessment of each patient and their tumour so as to be able to accurately define the extent and stage of disease as well as the suitability of an individual patient for a particular treatment approach.

1.3.2. Morbidity of radical radiotherapy

The reported rate of morbidity is dependent on registration, classification and grading, and most studies have limited information on this subject. Adverse effects can be difficult to distinguish from symptoms due to residual tumour, and may be influenced by irradiated volume, field arrangement, beam quality, fraction size, total dose, previous operative procedures in the area and the general condition of the patient (Abrahamsen *et al.*, 1990). Treatment related deaths are reported to be 2-3% (Goodman *et al.*, 1981; Hopkins *et al.*, 1987; Jahnson *et al.*, 1991). The side effects arise from damage caused to bladder and bowel included in the radiation field. The rate of any acute side effects is 24-70% for bowel symptoms (diarrhea, pain, and incontinence) and 53% for urinary symptoms (dysuria, frequency, pain, cramps) (Goffinet *et al.*, 1975). The actuarial rate of non-severe intestinal side effects according to RTOG scoring system is about 12% at 5 years. Severe or major events are usually defined as complications requiring surgery or at least 30 days of hospitalization. The rate of severe acute bowel complications is 2-17% (Duncan *et al.*, 1986; Goffinet *et al.*, 1975; Hopkins *et al.*, 1987; Sengelov *et al.*, 1997). Late effects occur after 3 or more months, with a median time of 18-26 months after completion of radiotherapy (Duncan *et al.*, 1986; Jahnson *et al.*, 1991). The crude rate of patients with major events such as ileus, peritonitis and fistula after radiotherapy is about 3.5-8% (Greven *et al.*, 1990; Salminen *et al.*, 1990; Shipley *et al.*, 1987). However, when using actuarial methods taking into consideration the number of patients at risk, the complication rates are as high as 11-79% (Bretheau *et al.*, 1996; Fossa *et al.*, 1993; Jahnson *et al.*, 1991; Pollack *et al.*, 1994; Sengelov *et al.*, 1997). Surgery performed for complication of late radiation effects can have a postoperative mortality as high as 33% (Jahnson *et al.*, 1991). The actuarial incidence of severe urinary radiation side effects, such as uraemia or contracted bladder is 8-12% at 5 years (Jahnson *et al.*, 1991; Sell *et al.*, 1991). In a prospective study by Lynch *et al.*, 1992,

symptoms and quality of life in 72 patients achieving complete response after radiotherapy was compared with a matched healthy non-irradiated control group. No significant difference in quality of life or treatment related symptoms except from haematuria was encountered. Information on late effects on sexual function is sparse. One study has found that 41% could practice normal coitus 18 months after radiotherapy (Sell *et al.*, 1991). Retrospectively, the frequency of morbidity has been similar for total doses in the range of 60-72 Gy and 50- 57.5 Gy (Quilty *et al.*, 1986). However, one study has found a reduced morbidity when the radiation dose was reduced from 55.0 to 52.5 Gy, but dose per fraction was also decreased (Whillis *et al.*, 1992). Data from several studies have shown that the morbidity increases with increasing field size (Davidson *et al.*, 1990) and increasing dose per fractionation (Abratt *et al.*, 1983; Fossa *et al.*, 1993; Jahnson *et al.*, 1991). The complication rate was higher with rotational Cobalt-60 irradiation than with two opposed fields (Shipley *et al.*, 1998) or with the four-field technique in retrospective comparisons (Jahnson *et al.*, 1991; Pollack *et al.*, 1994).

1.4 Prognostic markers

The search for biological parameters that could select patients who will respond to radiation treatment has become essential (Rodel *et al.*, 2000). The ultimate aim is to find individual markers related to stage and grade, sex, survival, recurrence and response to treatments. Such findings could be useful in routine clinical practice to determine which patients with muscle invasive bladder cancer should receive aggressive therapy, and in which patients the effectiveness of radiotherapy can be predicted.

1.4.1 Monoclonal mouse anti-human endothelial cell, CD 31 & CD34

Platelet/endothelial adhesion molecule (PECAM-1) or CD31 belong to the immunoglobulin superfamily with adhesive properties. It is a single chain membrane glycoprotein with a molecular mass of 130 kDa (Yan *et al.*, 1995; Parums *et al.*, 1990). CD31 is strongly expressed by all endothelial cells and more weakly on several types of leucocytes (Parums *et al.*, 1990). Functionally CD31 is an adhesion molecule with both homophilic and heterophilic binding. The homotypic binding involves interaction as an important step in leucocyte transendothelial migration (diapedesis) and passage into the extracellular matrix (Liao *et al.*, 1995). The heterotypic ligands have been reported to include integrin αvβ3 (Buckley *et al.*, 1996) and glycosaminoglycans (DeLisser *et al.*, 1993). Staining of vessels with antibody to CD31 has been shown to be appropriate for the assessment of angiogenesis in several types of tumours such as breast cancer (Horak *et al.*, 1992; Charpin *et al.*, 1995; Fox *et al.*, 1997), colorectal cancer (Takebayashi *et al.*, 1996; Engel *et al.*, 1996) and lung cancer (Giatromanolaki *et al.*, 1996).

The CD34 antigen is found on blood vessel endothelium, where it shows some variation in expression; most noticeably it appears to be absent from large veins and arteries and from sinuses in the placenta and spleen (Fina *et al.*, 1990; Kuzu *et al.*, 1992). On a subcellular level, CD34 is expressed on the luminal side and especially on membrane processes that interdigitate between endothelial cells (Fina *et al.*, 1990). On growing vascular sprouts, such as those seen on vessels in tumours, the location of CD34 is altered and is found on the luminal microprocesses of these vessels (Schlingemann *et al.*, 1990). Because the MY10 and QBEND10 antibodies react with oligosaccharide side chains (Sutherland *et al.*, 1992), it was important to show whether this resulted from expression of the CD34 gene in endothelial cells or simply reflected recognition of an oligosaccharide determinant present on an unrelated protein backbone molecule. Fina

and co-workers showed clearly that the CD34 gene is in fact expressed in endothelial cells (Fina *et al.*, 1990).

1.4.2 Monoclonal antibody Ki-67 antigen (MIB-1)

In recent years the study of the proliferative activity of bladder carcinoma has attracted the interest of numerous researchers involved in the search for an indicator of biological aggressiveness that might be more reliable than the classical prognostic factors, grading and staging. Markers used to assess cellular proliferation have included mitotic count, silver stained nucleolar organizer regions, Ki-67 and proliferating cell nuclear antigen. The 2 most promising immunohistochemical markers of cellular proliferation are Ki-67 and proliferating cell nuclear antigen. Ki-67 monoclonal antibody is currently used in evaluating the cellular turnover rate of malignant tumours (Brown *et al.*, 1990; Gerdes, 1990). It reacts with a non-histone nuclear protein of 395 kD and 345 kD expressed during G1, S-G2 and M phases, but not in GO phases of the cell cycle (Gerdes *et al.*, 1983; Gerdes *et al.*, 1984). The fraction of proliferating cells as determined by Ki-67 immunoreactivity is an important prognostic feature of many malignancies. Increased expression of these antigens indicates a higher level of proliferative activity in tumour cells, and is associated with tumours of more aggressive biological potential with an increased propensity for tumour progression and metastasis (Fradet *et al.*, 1993). Since this antibody was discovered in 1983 it has been extensively evaluated in a variety of human malignancies (Brown *et al.*, 1990). Okamura *et al.*, 1990, reported a correlation between increased expression of Ki-67 in bladder cancer and tumour grade and stage. Several other studies have not only confirmed this relationship but also have shown a significantly higher recurrence rate in surgically managed bladder tumours with a high proliferative index (Tsujihashi *et al.*, 1991; Bush *et al.*, 1991). The role of Ki-67 in

predicting local control after radiation is controversial. Lara *et al.*, 1998, found better local control after radical RT in bladder cancer patients with very low proliferating tumours, indicating that rapid tumour repopulation during fractionated radiotherapy might reduce the probability of tumour control. Conversely, in a study of Ogura *et al.*, 1995, a high proliferative index predicted radio-responsiveness in 60 patients treated with preoperative RT followed by radical cystectomy for invasive bladder cancer. Rodel *et al.*, 2000, also reported that the AI (apoptotic index) and the proliferation index (Ki-67) helped to identify patients whose invasive bladder tumour responded completely to a regimen of combined RCT (radiochemotherapy) following initial TUR-B. Five-year local control with bladder preservation was significantly better for tumours with a high AI and a high cell proliferation. In an exploratory multivariate analysis, these markers and especially their combination had a higher predictive value for initial CR and local control with preserved bladder than the T-stage, and the grading. Rodel *et al.*, 2000, concluded that patients with a high spontaneous AI and a high pretreatment Ki-67 index should be considered preferentially for treatment with RCT, whereas those with low proliferation and low levels of apoptosis are less likely to respond to RCT. Other studies also showed higher local control rates after definitive RT for head and neck cancer (Raybaud-Diogene *et al.*, 1997) and cervical carcinoma (Nakano *et al.*, 1997) with high Ki-67 labeling indices. These results are consistent with the clinical experience whereby rapidly proliferating tumours are more sensitive to irradiation than those growing slowly.

1.4.3 *Monoclonal mouse anti-human P53 protein*

The tumour suppressor gene product p53 is present in a wide variety of cells. In the normal cell the concentration of wild type p53 protein is generally below the detection

level of immunohistochemical methods because of its short half life (Vojtesek *et al.*, 1992). The p53 gene is located at 17p 13.1 (Isobe *et al.*, 1986) and codes for a 53 kDa (393 amino acid) transcription factor with a critical role in the regulation of DNA repair and apoptosis (Marx, 1993). It is not essential for life, but if both copies of the gene are knocked out in mice, there is a vastly increased risk of developing malignancy (Donehower *et al.*, 1992). Wild type (i.e. normal) p53 migrates to the nucleus in the S-phase of the cell cycle and switches on the transcription of WAF1 (p21), which bind and inhibits cyclin-dependent kinase 2 (CDK2) (Marx, 1993). CDK2 normally drives the cell into the next phase of the cycle and its inhibition allows time for DNA repair or orchestration of apoptosis. The p53 gene is a tumour suppressor gene (TSG). It can be inactivated by loss of both copies, by loss of one copy with mutation of the other, or by mutant copy acting in hemizygous state (Marshall, 1991). Mutated p53 protein has a much longer half-life than wild type, allowing its detection by immunocytochemical methods. The mutant form forms complexes with the wild type, prohibiting its function. Allelic losses at 17p are reported in many bladder cancers (Dalbagni *et al.*, 1993), and it has been shown that molecular defects at the DNA level correspond well with expression of mutant p53 (Esrig *et al.*, 1993). About half of bladder tumours show loss of p53 function and this has been shown to be strongly associated with higher tumour stage and grade (Reznikoff *et al.*, 1996). In superficial disease, although less frequent, mutant p53 expression is often associated with poor outcome (Tetu *et al.*, 1996). Also, Sarkis *et al.*, 1995, concluded that p53 has a role in predicting progression in superficial disease. Many p53 studies have been carried out in recent years and there have been conflicting reports. Not surprisingly, p53 has been investigated in all these fields. There seems no doubt that altered p53 expression is associated with higher stage, grade and poorer prognosis (Fujimoto *et al* 1992; Miyamoto *et al.*, 1996; Watanabe *et al.*, 1994). It is also suggested that p53 is a predictor of decreased survival, as Esrig *et al.*, 1994,

found that altered p53 expression was associated with a doubling in the risk of death, and other studies concur with this. Tsuji *et al.*, 1997, examined 31 primary TCCs, removed at radical cystectomy, for Ki-67 and p53 indices, they found that patients with tumours that had both a low Ki-67 index (< 32% of cells staining for the proliferative marker (Ki-67) and a low p53 index (< 20 % staining positive for p53) had no evidence of recurrence and none had died from bladder cancer at 66 months of follow-up, despite having muscle invasive disease. It was hoped that p53 might be of some use in preventing unnecessary radical cystectomy in patients who already had undetectable micrometastases. However, work by Glick *et al.*, 1996, showed that although altered p53 expression was very common in muscle invasive disease, it gave no predictive indication of which patients would eventually suffer recurrence or die after cystectomy. Again, there is controversy about the role of p53 in predicting recurrence, as work by two groups suggest p53 expression is associated with recurrence in superficial disease (Kuczyk *et al.*, 1995; Moch *et al.*, 1994), whereas a study of 86 tumours by Underwood *et al.*, 1996, showed no predictive relationship. The evidence is mounting that p53 has a significant role in progression and its frequent association with carcinoma in situ, and its obvious poorer outlook further substantiates this. This also supports the argument that p53 has a role in the path to progression from an early stage (Sarkis *et al.*, 1994; Schmitz-Drager *et al.*, 1994). A role exists for p53 in predicting poor response to chemotherapy and the possible avoidance of this form of therapy in favour of more aggressive intervention in patients with p53 positive tumours (Hudson *et al.*, 1995). Cote *et al.*, 1997, contradicted this when they found that tumours that were p53 positive responded three times more effectively to adjuvant chemotherapy, after radical cystectomy, than those that were negative. In a response to this article, Lowe and Jacks 1997 explained that these findings are not in keeping with most studies, and pointed out some factors that had caused such inconsistencies in the p53 studies, i.e. that

immunoreactivity does not necessarily reflect a tumour's p53 state, and that large studies looking at alterations at the DNA level might resolve the discrepancies. An interesting study by Raitanen *et al.*, 1997, provided evidence that DNA aneuploidy precedes altered p53 expression and that some tumours change from negative to positive expression at recurrence. This type of longitudinal study provides valuable information about tumour progression over time, and it is clear that there are many other pathways to progression that do not rely on p53. p53 is the most important discovery in tumour molecular biology to date, but disappointingly, despite a decade of work, no effective targeting therapy has been discovered.

1.4.4 Monoclonal mouse anti-human Bcl-2 Oncoprotein

Recent advances in molecular genetic analysis have shown that the genetic alterations in oncogenes and suppressor genes, bcl-2, p53 and c-*myc*, appear to influence the susceptibility of cells to apoptosis. This might influence the sensitivity of cancer cells to radiation and chemotherapy (Hale *et al.*, 1996). It has been estimated that more than half of the world's cancer burden is composed of malignancies with an alteration in the tumour suppressor gene locus or in proto-oncogenes or both (Cohen and Ellwein 1991). All of these changes may confer a growth advantage to the tumour cells by stimulating cell proliferation. However, tumour growth (i.e. the rate of cell accumulation) is dependent not only on cell proliferation but also on the rate of physiologically occurring cell death. Thus, it is conceivable that neoplastic growth also may be caused or promoted by factors inhibiting cell death (Kerr *et al.*, 1994; Reed 1994). A new class of proto-oncogenes has been defined that contribute to malignancy by inhibiting programmed cell death or apoptosis (Williams, 1991). The prototype of proteins that is encoded by these oncogenes and involved in such a regulatory pathway is the bcl-2 protein (Tsujimoto *et al.*, 1985). The bcl-2 gene was initially cloned from the t(14;18)

translocation in a follicular B-cell lymphoma (Tsujimoto *et al.*, 1985). It is over expressed in various human cancers, indicating a close association with tumour development and progression (Fontanini *et al.*, 1995; Bhargava *et al.*, 1994; Kapucuoglu *et al.*, 1997). This gene protects against apoptosis induced by numerous apoptotic stimuli including growth factor withdrawal, irradiation, glucorticoids and multiple chemotherapeutic agents, to prolong cell survival (Nunez *et al.*, 1990; Chen *et al.*, 1995; Alnemri *et al.*, 1992; Miyashita *et al.*, 1993; Chiou *et al.*, 1994). Although its biochemical mechanism is incompletely understood, bcl-2 protein appears to control a distal step that may be a final common pathway involved in apoptosis. Because of its anti-apoptotic effects, bcl-2 protein is thought to be an important multidrug resistance molecule. Overexpression of bcl-2 protein has been shown to provide protection against a wide variety of apoptotic insults, including radiation and nearly all chemotherapeutic drugs (an issue that relates to the tumour cell metastatic propensity) (Ruoslaht and Reed, 1994; Reed, 1995). It has also been reported that cells that coexpress bcl-2 can override the apoptotic death triggered by wild-type p53 by excluding the p53 protein from the nucleus, thus being allowed to proliferate (Ryan *et al.*, 1994).

Bcl-2 is a member of a family of bcl-2 homologues that regulate apoptosis differently. In this multigene family some members (such as bcl-2, bcl-XL and bag-1) block cell death (Reed, 1994; Boise *et al.*, 1993; Walton *et al.*, 1993; Takyama *et al.*, 1995), whereas others promote apoptosis. The latter include bax, bad, bak and bcl-XS (Reed, 1994; Oltvai *et al.*, 1993; Farrow *et al.*, 1995; Yang *et al.*, 1995). The bax gene has extensive amino acid homology with bcl-2, and it may form heterodimers with bcl-2 that oppose bcl-2 function and contribute to cell death. It has been proposed that the ratio of bcl-2/bax and other anti-apoptotic members of the bcl-2 family govern the relative sensitivity response of cells to apoptotic stimuli (Oltvai *et al.*, 1993; Korsmeyer *et al.*, 1993). The tumour suppressor gene p53 that is frequently mutated in human cancers

also regulates cell death. Cells with mutated p53 are more resistant to chemotherapy because of a lesser ability to undergo apoptosis. Clinical studies show that p53 mutated tumours are more resistant to chemotherapy than p53 wild-type tumours. Miyashita *et al.*, 1994 reported that wild-type p53 protein directly contributes to the negative regulation of bcl-2 and positive regulation of bax gene expression (Miyashita *et al.*, 1994). The bcl-2 gene may block p53 induced apoptosis, suggesting that bcl-2 and p53 may participate in a common pathway to regulate cell life and death (Wang *et al.*, 1993). Gazzaniga *et al.*, 1996, evaluated the expression of bcl-2 and bax in low grade bladder cancer and found that bcl-2/bax messenger RNA expression may be a marker for disease, early relapse and progression. The increased expression of bcl-2 has been found to alter tumour sensitivity to chemotherapy and radiation (Dole *et al.*, 1994; Pollack *et al.*, 1997). Pollack *et al.*, 1997, reported that bcl-2 overexpression was associated with impaired radiation response in patients with bladder cancer treated with a regimen of preoperative radiotherapy. The significance of this anti-apoptotic protein, as well as the role of proliferation as measured by the Ki-67-labeling index in predicting local control in bladder cancer treated by a combination of radio- and chemotherapy, remains to be established. Correlating the expression of several molecular markers and clinicopathological factors and elucidating their interrelationships may finally help to identify subgroups of patients that will most probably benefit from bladder preservation by combined modality treatment.

1.4.5 Monoclonal mouse anti-human macrophage, CD 68

Macrophages belong to the mononuclear phagocyte system. They form a heterogeneous cell population because of different developmental and functional stages (Rutherford *et al.*, 1993). Macrophages share a common progenitor cell with granulocytes in the bone

marrow. Commitment to the macrophage lineage gives rise to the macrophage colony-forming cells, which are succeeded by monoblasts, the first characteristic phagocytic cells. They further differentiate into promonocytes and bone marrow monocytes (Rutherford *et al.*, 1993; Furth *et al.*, 1992). Monocytes then enter the blood stream and migrate into tissues, where they undergo final differentiation to tissue macrophages, often assuming tissue-specific properties as histiocytes, alveolar macrophages, Kupffer cells, osteoclasts, peritoneal macrophages, synovia type A cells, or microglia. Developmental stages and differentiation steps of macrophages have been defined according to the intracellular localization pattern of peroxidase activity (Furth *et al.*, 1992) or the expression of distinct antigens. (Sorg *et al.*, 1992) Subsequently, it was shown that inflammatory infiltrates contain several differentiation stages of macrophages (Rutherford *et al.*, 1993; Furth *et al.*, 1992; Sorg *et al.*, 1992). Macrophages are thus present ubiquitously in all tissues and in case of inflammation certain subtypes are recruited from the blood-borne monocytes. A multitude of functions can be performed by the mononuclear phagocyte system, such as endocytosis, cytotoxicity, and secretion of more than 100 cell products (Adams *et al.*, 1992; Nathan, 1987). However, macrophages do not carry out all these functions at the same time. Their activities are rather dependent on the pathophysiological situation (Rutherford *et al.*, 1993; Adams *et al.*, 1992). In order to become angiogenic, the versatile secretory potential of macrophages needs to be activated. Tumour angiogenesis was initially believed to be induced only by tumour cells themselves (Folkman, 1985; Blood *et al.*, 1990). However, macrophages also appeared to be involved in vascularization and growth of tumours. Mice depleted of monocytes showed a strong reduction of tumour vascularization in implanted syngeneic fibrosarcomas (Evans, 1977). Similarly, tumour growth in nude mice showed association with the monocytic infiltrate of tumours (Stenzinger *et al.*, 1983). Neoplastic tissues exhibited angiogenic activity in vivo and in

vitro only when macrophages were present (Mostafa *et al.*, 1980; Polverini *et al* 1984). Polverini and Leibovich, 1984, showed that macrophages isolated from tumours were indeed able to induce neovascularization in the cornea assay. Thus, vascularization of tumours appears to be either induced or modulated by macrophages. Their appearance at sites of neoplastic growth is part of the inflammatory reaction usually provoked by tumours (Pugh-Humphreys 1992). Macrophages are set into different functional states by a process called activation. This term was originally introduced to describe increased phagocytic or microbicidal activities of macrophages induced by a given stimulus such as lipopolysaccharide (LPS) (Adams *et al.*, 1992). Later, activation of macrophages was also thought to entail alterations of their chemotactic response and their secretory activity (Adams *et al.*, 1992). However, activation does not lead to simultaneous enhancement of all macrophage activities. When activated macrophages increase performance of particular functions, they down-regulate others. For example, certain macrophages can be either tumouricidal or bactericidal, but not both at the same time (Rutherford *et al.*, 1993). Thus, the activation process appears to be based on complex regulation with different stimuli involved. Activation of macrophages proceeds in sequential steps (Rutherford *et al.*, 1993; Adams *et al.*, 1992). In order to present antigens, for example, macrophages first have to express class II major histocompatibility complex (MHC) molecules followed by expression of interleukin-l (IL-l) or other cytokines (Steinmann 1988). Activation sequences for other macrophage functions such as the induction of angiogenesis have not yet been elaborated.

Several studies have confirmed that macrophages need to be activated to exert angiogenic activity (Steinmann, 1988). Human and murine monocytes did not promote neovascularization unless treated with concanavalin A, endotoxin, or other activators (Koch *et al.*, 1986; Kaminski *et al.*, 1987; Meyer *et al.*, 1989). Activation of macrophages in angiogenesis has biological relevance. The proliferation rate of

endothelial cells in normal tissues is very low (Hobson *et al.*, 1984). In growth and repair processes neovascularization is up regulated for brief periods and then completely inhibited or even down regulated. Persistent neovascularization, on the other hand, is a characteristic feature of malignant tumours and chronic diseases, such as rheumatoid arthritis. Thus, in healthy organisms angiogenesis is under tight control. Cells able to induce neovascularization should be expected to remain inactive unless activated. Many of the macrophage derived angiogenic factors are indeed synthesized or released only by activated macrophages. Among the potential activators of macrophages, the bacterial product LPS is known to induce angiogenic activity (Koch *et al.*, 1986; Polverini *et al.*, 1977) but does not appear to be a general stimulus of angiogenesis. More specific activation signals could be provided by the particular metabolic conditions found in wounds. Macrophages became angiogenic when exposed to low oxygen tensions (Knighton *et al.*, 1983) or to woundlike concentrations of lactate, pyruvate, or hydrogen ions (Jensen *et al.*, 1986). Macrophages can also be activated by cytokines such as interferon-γ (IFN-γ), granulocyte-macrophage colony-stimulating factor (GM-CSF), platelet-activating factor (PAF), or monocyte chemotactic protein (Adams *et al.*, 1992). These activating cytokines are released by a number of cells, among them activated endothelial cells (Goerdt *et al.*, 1993). An attractive model for the controlled activation of macrophages at sites of inflammation would therefore be the activation by local endothelial cells. Macrophages recruited to sites of inflammation by endothelial adhesion molecules (Goerdt *et al.*, 1993) thus become simultaneously activated by endothelial cytokines such as GM-CSF, PAF, and monocyte chemotactic protein.

In invasive breast carcinoma, the neoplastic cell population is often outnumbered by such stromal cells as tumour associated macrophages (TAMs), which can comprise more than 50% of the total tumour mass (O'Sullivan *et al.*, 1994). It is thought that monocytes in the peripheral circulation are recruited to the tumour site by the release of

the chemotactic cytokines, monocyte chemotactic protein-1 (MCP-1) (Graves *et al.*, 1991), CSF-I (Scholl *et al.*, 1994), granulocyte macrophage CSF (Fu *et al.*, 1992), and VEGF (Clauss *et al.*, 1990) by such tumours. Once recruited, monocytes differentiate to become TAMs and are modified in the tumour microenvironment to secrete several growth factors, such as EGF (O'Sullivan *et al.*, 1993), TNF-∝ (Pusztai *et al.*, 1994), VEGF and bFGF (Lewis *et al.*, 1995). Focal macrophage infiltration also appears to be an important prognostic factor in invasive carcinoma of the breast, being predictive of a worsened outcome and of reduced relapse-free and overall survival when MI (macrophage index) is high. Leek *et al.*, 1996, also reported that MI is a more powerful predictor of survival than nodal status, and suggested that macrophages may have a direct role in disease progression. Other studies have found associations between higher levels of macrophage infiltration and markers of poor prognosis such as Ki-67 (Hildenbrand *et al.*, 1995), and Pupa *et al.*, 1996, have described a positive correlation between macrophage infiltration and c-erbB2 as well as high grade in invasive breast carcinoma. Herrmann *et al.*, 1994, also demonstrated a significant correlation between CD68-positive cell infiltration and vascularization in thyroid carcinoma, enumerating the infiltrating cells as the possible source of angiogenic factors. In a more recent study, Hanada *et al.*, 2000, reported on series of 63 patients with bladder cancer, including 40 superficial bladder cancers and 23 invasive bladder cancers. The TAM count in invasive bladder cancers was significantly higher than in superficial bladder cancers. The microvessel count (MVC) in invasive bladder cancers was also significantly higher than in superficial bladder cancers. There was a positive correlation between TAM count and MVC. Patients with a high TAM count (≥67) showed significantly higher rates of cystectomy, distant metastasis and vascular invasion than those with a lower TAM count (<67). The 5-year survival rate was significantly lower in patients with a high TAM count than those with a low TAM count.

The best macrophage reagents produced to date are those recognising the CD68 antigen (Knapp, 1989). This 110 kD antigen belongs to a family of acidic, highly glycosylated lysosomal glycoproteins that include the lamp-1 and lamp-2 molecules (Fukuda, 1991). CD68 is the human homologue of the murine macrosialin antigen (Holness *et al.*, 1993) and is present in the cytoplasmic granules of monocytes, macrophages, neutrophils, basophils and large lymphocytes (Pulford *et al.*, 1990). This antigen is also expressed to some degree in the cytoplasm of some non-hemopoietic tissue. The function of the molecule is currently unknown. The murine PG-M1 monoclonal antibody (IgG3, k) was raised against spleen cells of Gauchers disease (Falini *et al.*, 1993). Reactivity with cells transfected with a human cDNA encoding for the CD68 antigen confirms PG-M1 as a member of the CD68 cluster. In bone marrow paraffin sections, PG-M1 strongly stains macrophages but not granulocytes and myeloid precursor. PG-M1 also shows immunopositivity with mast cells and synovial cells.

1.5 Aims of the study

1.5.1 General objective

The purpose of this study was to investigate the role of several biologic markers that may predict response to radiotherapy in muscle invasive bladder transitional cell carcinoma. This study examines the relationship of clinical data and histopathological parameters in cases of muscle invasive bladder TCC with response to radiotherapy. It involves clinical audit and utilises histological skills including tumour recognition and grading, immunohistochemistry and the use of a Chalkley eyepiece graticule, as well as statistics to determine treatment and outcome. This study may indicate predictive factors that would alter patient's treatment and management.

1.5.2. Specific objectives

To retrospectively determine the relationship between the following:

a) Patient's age, sex, and haemoglobin level and the response to radiotherapy.

b) Tumour stage, grade and the response to radiotherapy.

c) Tumour angiogenesis (CD31&CD34) in bladder TCC and the response to radiotherapy.

d) Intratumour macrophage (CD68) concentration and the response to radiotherapy.

e) Tumour proliferation as measured by Ki-67 (MIB1) and the response to radiotherapy.

f) p53 expression and apoptosis (bcl-2) of tumour cells and the response to radiotherapy.

CHAPTER TWO

MATERIALS & METHODS

MATERIALS & METHODS

2.1. Patients

Muscle invasive transitional cell carcinomas from 101 patients were identified from the archives of Derriford Hospital, Plymouth and Royal Devon & Exeter Hospital, Exeter between 1984-1996.

The initial material was reviewed, and this consisted of either bladder biopsies or specimens from transurethral resections of the bladder tumour (TURBT) taken before any treatment had been started. All cases were reviewed. Either, the original haematoxylin and eosin (H&E) sections were examined if possible or, alternatively, 4 μm thick H+E stained recuts were examined. Muscle invasive transitional cell carcinoma was confirmed only when there was unequivocal invasion through bundles of muscularis propria by tumour (Fig. 2.1). All equivocal cases were excluded. Similarly, cases of non transitional cell carcinoma, including adenocarcinoma, squamous cell carcinoma, or small-cell carcinoma were also excluded.

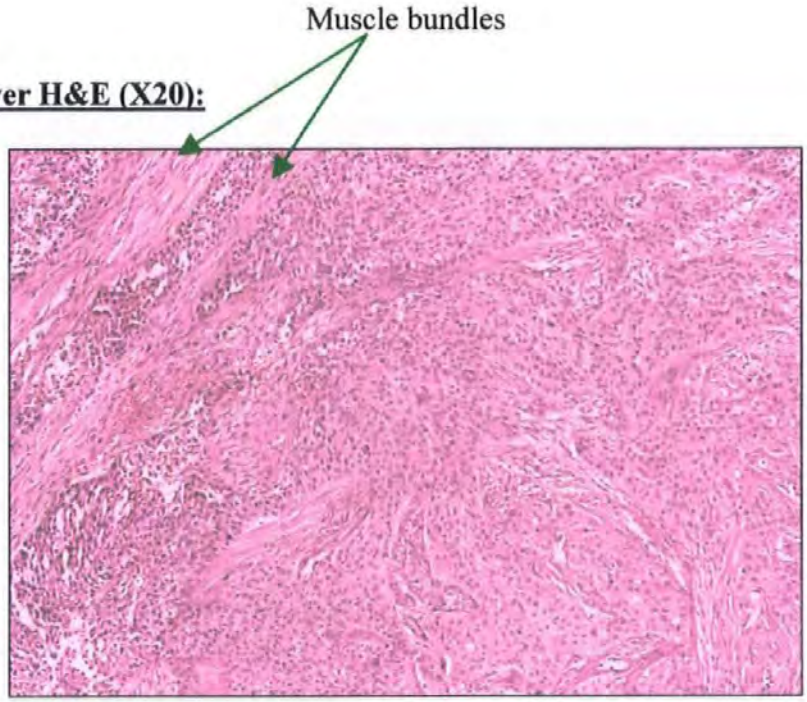
Each tumour was graded according to the World Health Organization (WHO 1982) grading system and staged according to the International Union Against cancer (1997) TNM classification. All cases were treated by one regime of radiotherapy with no adjuvant or concomitant chemotherapy.

After completion of the radiotherapy course, all the patients were followed up closely by cystoscopy and other complementary examinations as clinically indicated. The follow-up regime required at least cystoscopy to be performed every 3 months for the first 2 years, every 6 months from the third to the fifth year, and yearly from then on.

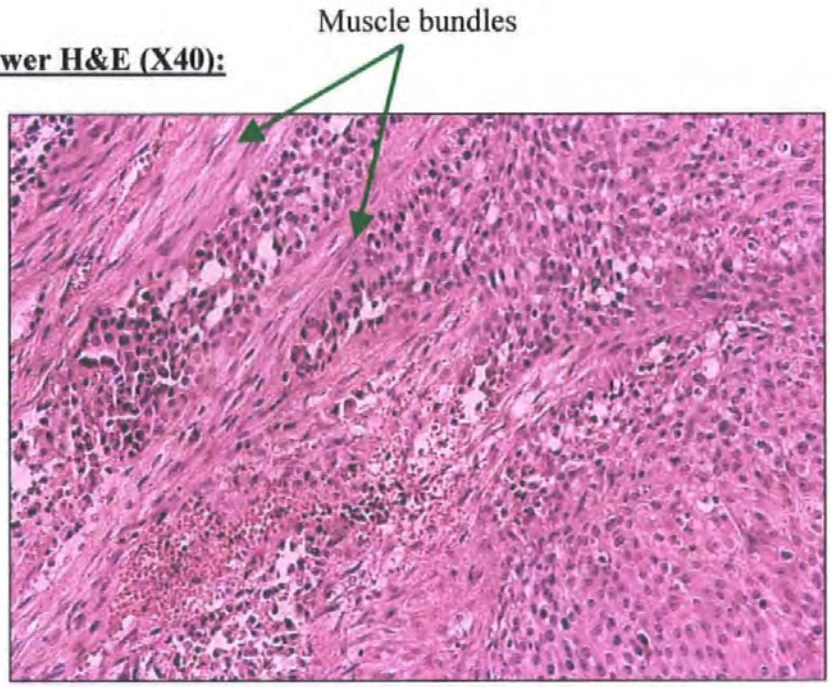
All the patients' records were examined for the recorded post-radiotherapy tumour response.

Fig. 2.1. Muscle invasive TCC:

a) Low power H&E (X20):



b) High power H&E (X40):



The patients were classified into two groups, in two different ways, according to the response; the first group includes (1) those free of disease (no tumour detected in the bladder at the 3-month cystoscopy), (2) those with residual disease (tumour present in the bladder at 3-months cystoscopy). The second classification includes (1) those with persistent or recurrent cancer in the bladder (tumour recurred after an initial 3 months negative check cystoscopy together with patients with residual disease at 3 months), (2) those free of disease in the bladder at all subsequent cystoscopies.

From the clinical records, data relating to the position of the primary tumour in the bladder, age, stage, grade and radiotherapy dose including number of fractions and number of days over which the course of radiotherapy was given. Patient survival was recorded for analysis.

2.2 Immunohistochemistry

2.2.1 Reagents

For each case, one representative formalin-fixed, paraffin wax-embedded block was chosen from between 1 and 3 different block sections. Serial slices, each 4μm thick were taken from each chosen block and were immunostained using the antibodies previously described. In brief, the antibodies used were CD31 (Monoclonal Mouse Anti-Human Endothelial cell CD31, Clone JC/70A, Code No. M 0823, DAKO, Denmark.), CD34 (Anti-Endothelial cell Marker, QBEnd10-Mouse Monoclonal immunoglobulin G, sub-class 1, code No. M 87030, Bionostics, UK.), MIB1-KI67 (Monoclonal antibody Ki-67 Antigen, Clone MIB-1, Immunotech, France), bcl-2 (Monoclonal Mouse Anti-Human BCL2 Oncoprotein, Clone 124, code No. M 0887, DAKO, Denmark), CD68 macrophage (Monoclonal Mouse Anti-Human Macrophage, CD68, Clone PG-M1, Code No. M 0876, DAKO, Denmark), and p53 (Monoclonal Mouse Anti-Human p53 Protein, Clone DO-7, Code No. M 7001, DAKO, Denmark).

2.2.2 Immunohistochemistry procedure

Below outlines the immunohistochemical technique used for all the antibodies, with indication of specific techniques required for certain antibodies. To maximize homogeneity of staining for each antibody, immunostaining for individual antibodies was performed in batches with positive and negative control slides included in every batch. The positive control tissue was human tonsil. The negative control was bladder tissue, which was treated with Tris/Tween buffer only in the absence of primary antibody.

1. Paraffin sections for immunoperoxidase, were cut at 4 microns thick, onto APES (3-aminopropyltriethoxysilane, Sigma code A3648) coated slides and placed in a 37 ° C incubator and left overnight. The following morning, the slides were transferred to a 60 ° C incubator for one hour to complete the drying process.
2. Slides were de-waxed in :

   Xylene 1 minute

   Xylene 1 minute

   Xylene 1 minute

3. Xylene removed in :

   Alcohol 1 minute

   Alcohol 1 minute

   Alcohol 1 minute

4. Endogenous peroxidase was quenched with (3%) hydrogen peroxide solution in methanol for 10 minutes (9 mls 30% hydrogen peroxide to 300 mls of methanol).
5. Sections were washed with running tap water for 5 minutes.
6. The antigen retrieval method used depended on the antibody, and this included microwave oven and pressure cooking.

   Sections for CD31, bcl-2 and p53 immunostaining were placed into 800ml of citrate buffer (pH 6.0), and microwaved at full power in a 950W microwave oven, for 30 minutes.

   Sections for MIB1 staining were pressure cooked for 1½ minute at full pressure in citrate buffer (pH 6.0).

   Following these treatments, the hot buffer was gradually replaced by cold running tap water to cool the sections.

Sections for CD68 immunostaining were treated with 0.1% calcium chloride solution at 37 ° C for 10 minutes, followed by washing in cold running tap water for 5 minutes.

7. The sections were ringed with a PAP pen. This acts as a hydrophobic barrier to prevent the antibody solutions from spreading over the entire slide. Slides were then placed in a bath of Tris (Hydroxy methyl methylamine, BDH Laboratory supplies) and Tween 20 (polyoxyethylene (20) sorbitan monolaurate BDH) buffer (pH 7.6) for 5 minutes. Tween 20 is a detergent added at 0.05% to decrease background staining.
8. The excess buffer was drained off and Normal Horse Serum (NHS) diluted 1:100 in Tris/Tween buffer was pipetted onto the sections and incubated for 20 minutes at room temperature. This reduced non-specific binding of the antibody to the tissue.
9. The excess NHS was drained off and the diluted primary antibody, at the dilution given below was applied to the sections.

   The optimum dilutions for each primary antibody had been carefully titrated for this immunohistochemistry regime. Incubation was for 30 minutes at room temperature.

Dilution for antibodies

Primary Dilution for CD31: 1:40

Primary Dilution for CD34: 1:40

Primary Dilution for CD68: 1:40

Primary Dilution for MIB1: 1:40

Primary Dilution for bcl-2 : 1:20

Primary Dilution for p53: 1:300

10 The excess antibody was then drained from the sections, which were then placed in a Tris/Tween buffer bath for 5 minutes. Two further changes of buffer were performed to wash off excess unreacted antibody.

11 The buffer was drained off and the secondary antibody applied. The secondary antibody was a biotinylated universal horse antibody, which reacts either with mouse or rabbit primary antibodies. This was diluted 1:50 in 1:100 Normal Horse Serum. Incubation was for 30 minutes at room temperature.

12 The sections were washed as in step 10.

13 The buffer was drained off and the tertiary antibody applied. The tertiary antibody was a streptavidin-biotin complex (Vectastain Universal Elite ABC Kit, Cat. No. PK-6200, Vector Laboratories, USA) prepared by mixing solution A (Streptavidin) with an equal amount of solution B (Biotinylated horseradish peroxidase) in Tris/Tween buffer to give a 1:50 dilution. Incubation was for 30 minutes at room temperature.

14 The working DAB (3,3-diaminobenzidine tetrahydrochloride) solution was prepared 10 minutes before the end of the incubation of the tertiary antibody, using Sigma tablets (Sigma Fast, D4293). One tablet of DAB and one tablet of urea hydrogen peroxide were added to 15 ml of distilled water and allowed to dissolve.

15 The sections were washed as in step 10.

16 The filtered DAB solution was applied to sections for 10 minutes.

17 The excess DAB was tipped into a bleach container to deactivate excess DAB and slides were placed in a slide rack.

18 Slides were washed in running water for 1 minute.

19 Slides were placed in DAB enhancing solution for 2 minutes; DAB enhancing solution contains Copper Sulphate and Sodium Chloride Salt and makes the

DAB appear darker brown.

20 Slides were washed in tap water and placed in haematoxylin for 2 minutes.

21 Slides were washed in tap water then differentiated haematoxylin in acid alcohol (0.7% concentration).

22 Slides were washed in tap water.

23 Slides were then "blued" in Scotts tap water substitute for 10 seconds.

24 Slides were washed in tap water.

25 Slides were dehydrated in serial alcohol (industrial methylated spirit),

Alcohol 1min

Alcohol 1 min

Alcohol 1 min

26 Slides were bathed in serial xylenes

Xylene 1min

Xylene 1min

Xylene 1min

27 All sections were hand mounted using DPX mounting medium (RA lamb) and glass coverslips.

All immunostained slides were analysed and scored in a blind fashion without knowledge of outcome, grade, stage or survival data by one observer (Dr. Z. Saki) for consistency

2.2.3 Vessel density determinations

Antibodies CD31 and CD34 were used to detect the endothelial cells of the microvasculature. This includes capillaries and small venules. Both these antibodies are well known vascular markers and anti CD34 antibodies have been found to recognize small-calibre vessels that are associated with neovascularisation in bladder cancer more effectively than factor VIII antibodies (Bochner *et al.*, 1997). The techniques described by Weidner *et al.*, 1991 and Fox *et al.*, 1995, outlined the assessment of microvessel density.

Examination of immunostaining by CD31 (Fig. 2.2) and CD34 (Fig. 2.3), the microvessel density was assessed in areas with a solid tumour morphology away from any distorting artefact (Fox *et al.*, 1995a).

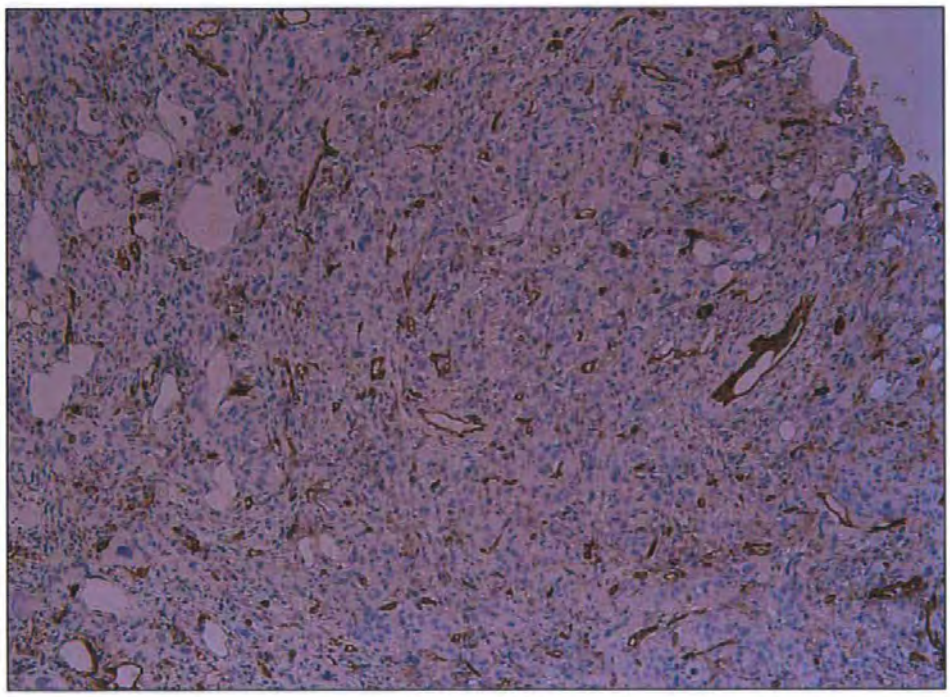
In brief, the vessel counting was performed in areas of maximal neovascularization; these so-called "hotspots" were areas where the highest numbers of discrete microvessels were stained and were identified after scanning the entire section at low power (X10) by light microscopy (Leitz, Laborlux 12, Germany). Significant heterogeneity of vessel density was noted in virtually all cases. Areas associated with ulceration or granulation tissue were excluded.

Any brown-staining endothelial cell or endothelial cell cluster, clearly separated from an adjacent microvessel, was regarded as a single, countable microvessel. Vessel counts were then estimated using a 25-point Chalkley eyepiece graticule (Chalkley, 1943) at X40 magnification. The graticule (Graticules, Tonbridge, UK) was rotated in the

eyepiece to where the maximum number of graticule dots overlay immunohistochemically identified vessels or their lumens. The mean of the three Chalkley count was used for the individual tumour. This method of vascular assessment has been previously shown to correlate reproducibly with field counts in invasive breast carcinomas (Fox *et al.*, 1995a).

Fig. 2.2. Staining endothelium cells for CD31:

a) **Low power:** CD31 positive immunostaining outlining the microvasculature in tumour tissue.



b) **High power (X40):** Arrows show CD31 positively stained microvessels.

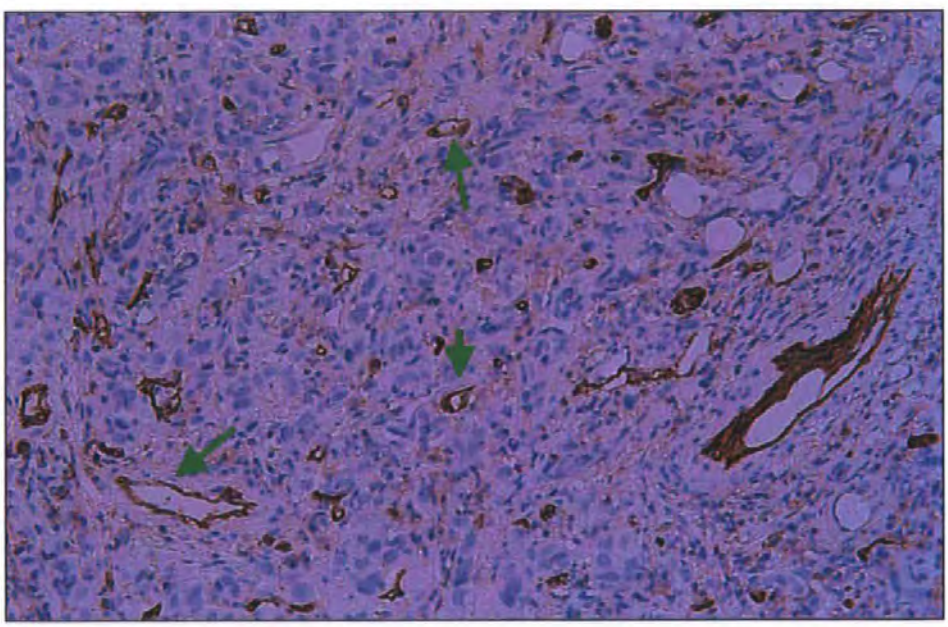
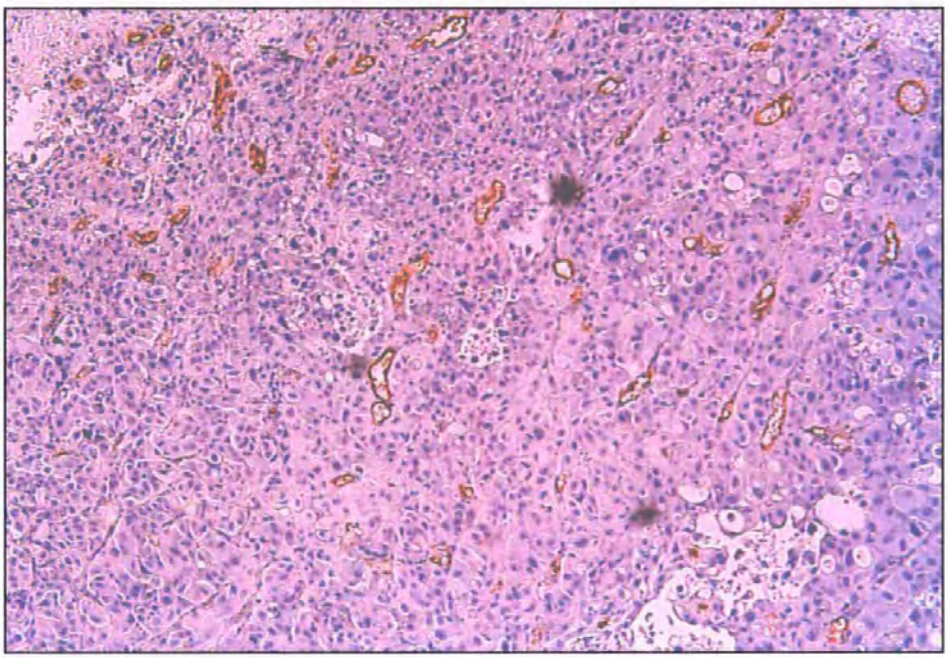
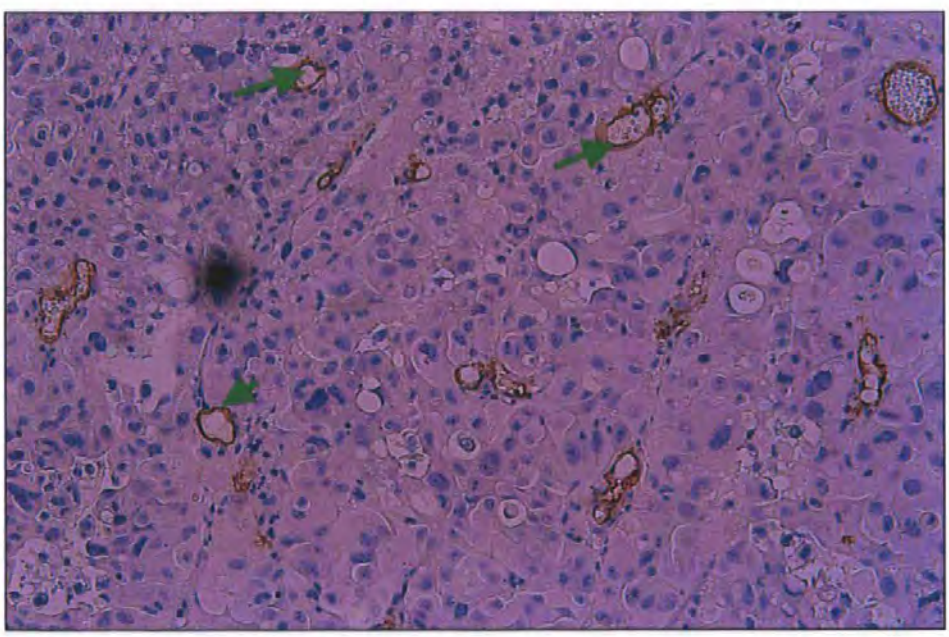


Fig. 2.3. Staining endothelium cells for CD34:

b) Low power: Numerous CD34 positive immunostaining microvessels are visible in the tumour tissue.



b) High power (X40): Arrows show CD34 positively stained microvessels.



2.2.4 Immunohistochemical analysis of bcl-2, p53, MIB-1 and CD68.

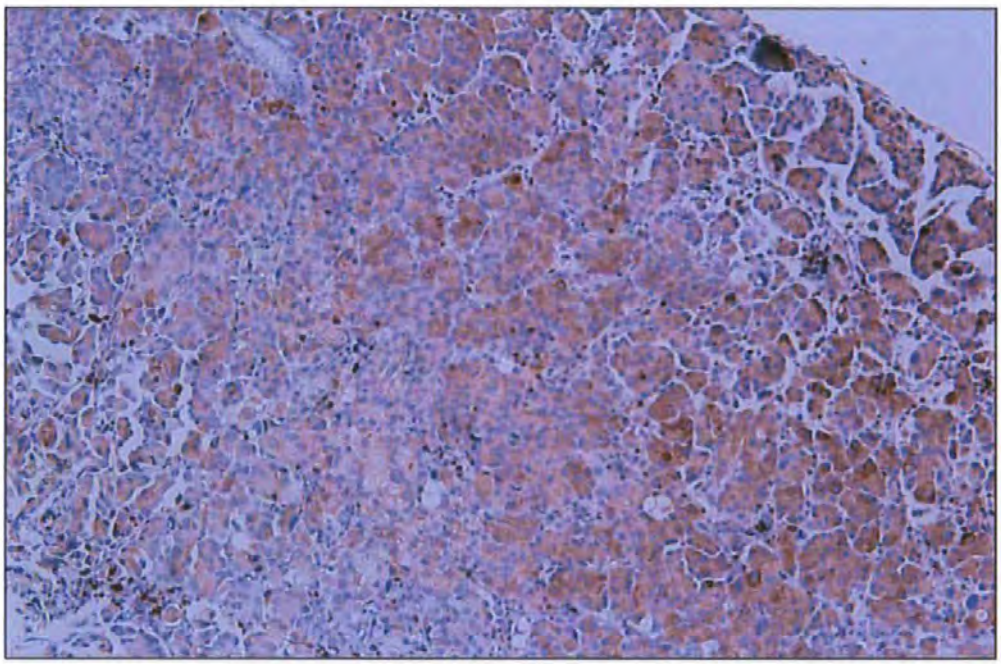
The expression of bcl-2 was determined to be positive when there was strong or weak (focal or diffuse) cytoplasmic staining. On the contrary, when there was no cytoplasmic staining, bcl-2 was scored as negative. Figure 2.4 shows positive expression of bcl-2 in muscle invasive bladder transitional cell carcinoma.

The sections for p53 (Fig. 2.5) and MIB-1 (Fig. 2.6) were scanned for areas with the highest density of positively stained nuclei (strong brown nuclear staining). In many of the sections there were varying degrees of nuclear staining. However, every cell that showed intense and weak nuclear staining was considered positive. MIB-1 proliferation and p53 protein staining were not classified as positive or negative. Rather, their degree of staining was recorded as a labelling index (LI). A similar method was applied for assessing the concentration of tumour macrophages, stained by CD68 (Fig. 2.7 a & Fig. 2.7 b). Tumor regions were examined by scanning at low magnification (X10) (Leitz microscope), and counts (of approximately 1000 cells) were then estimated using a 10x10 square grid eyepiece graticule (Jencons-PLS, Cherrycourt way, Stanbridge Rd, Leighton Buzzard, LU7 8UA, UK) (Fig. 2.8a) at X40 magnification. The counting was repeated 2 times for each slide. The labelling index was determined as the percentage of positive nuclei out of the total number of nuclei examined. The average score was taken for each case.

An alternative method of assessing p53 immunostaining was also applied. Tumour was graded positive if there was > 30% positive immunostaining of nuclei and were graded negative if there was <30% positive immunostaining nuclei (Jahnson *et al.*, 1995). There was no significantly difference between this and the counting system used above (see appendix page 193 & 199).

Fig. 2.4. Bcl-2 positive staining:

b) Low power: This shows positive cytoplasmic staining for bcl-2.



a) High power (X40): This shows positive cytoplasmic staining for bcl-2.

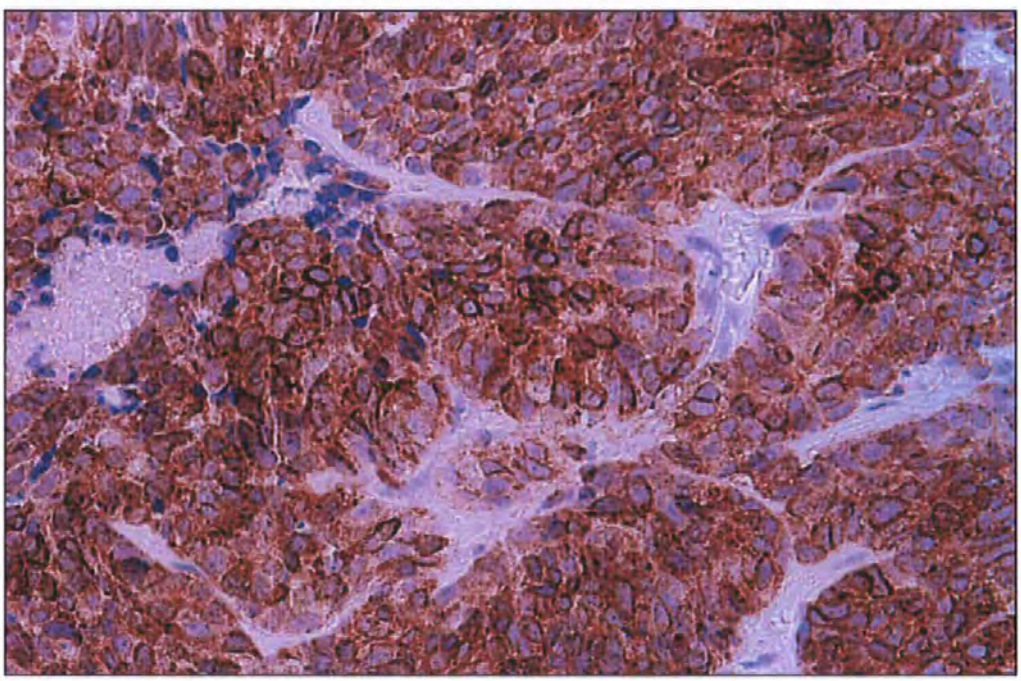
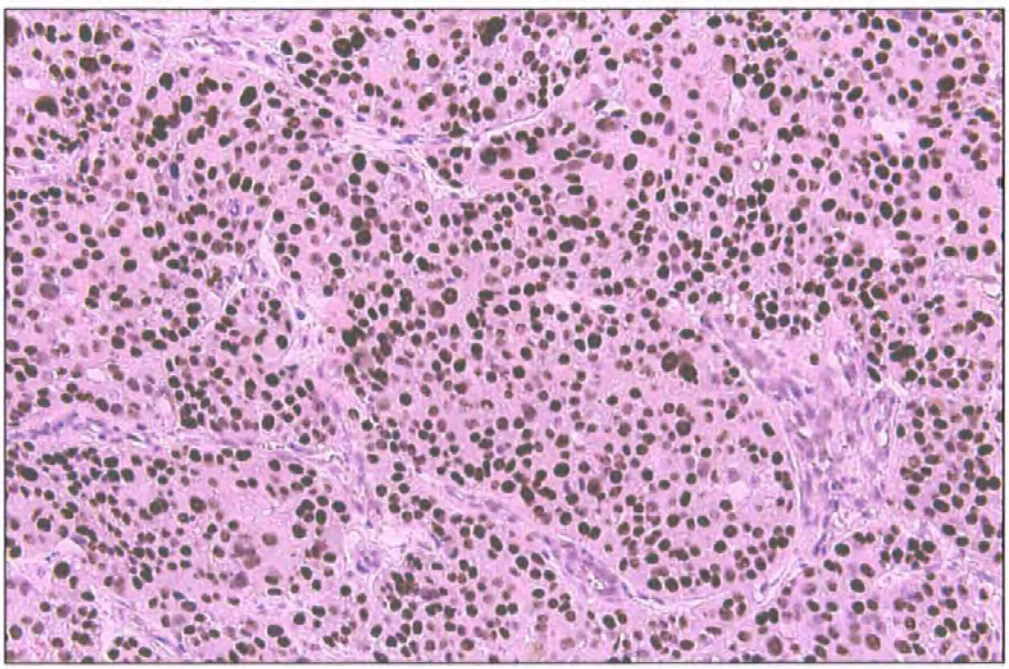


Fig. 2.5. p53 positive staining:

a) Low power: area with the highest density of positively stained nuclei for p53.



b) High power (X40): arrows show positive nuclear staining for p53.

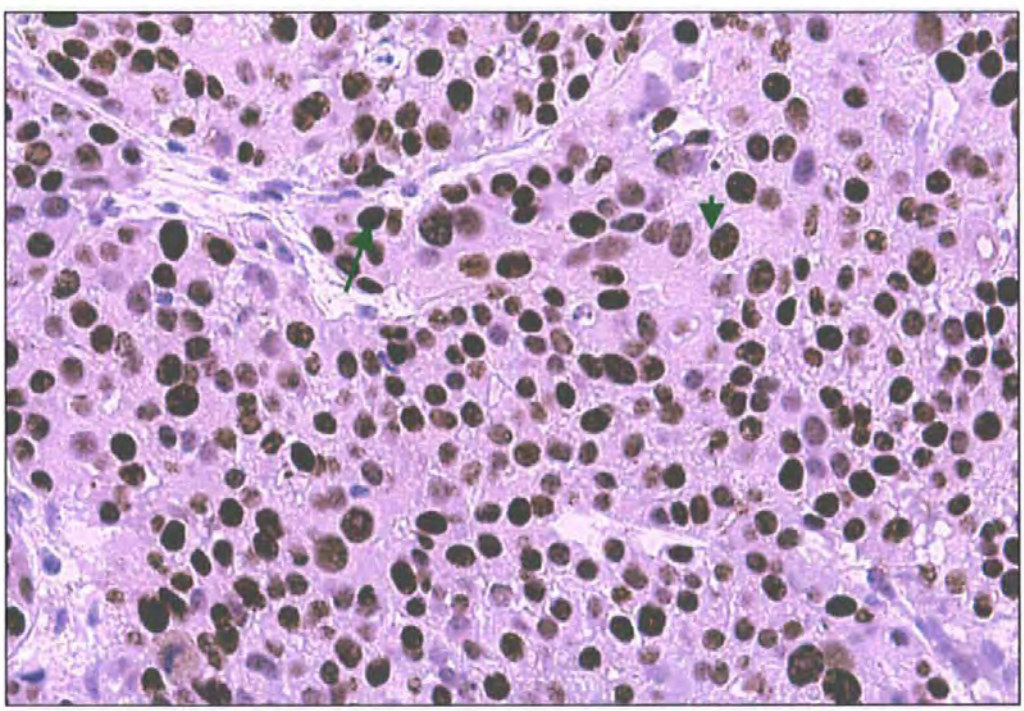
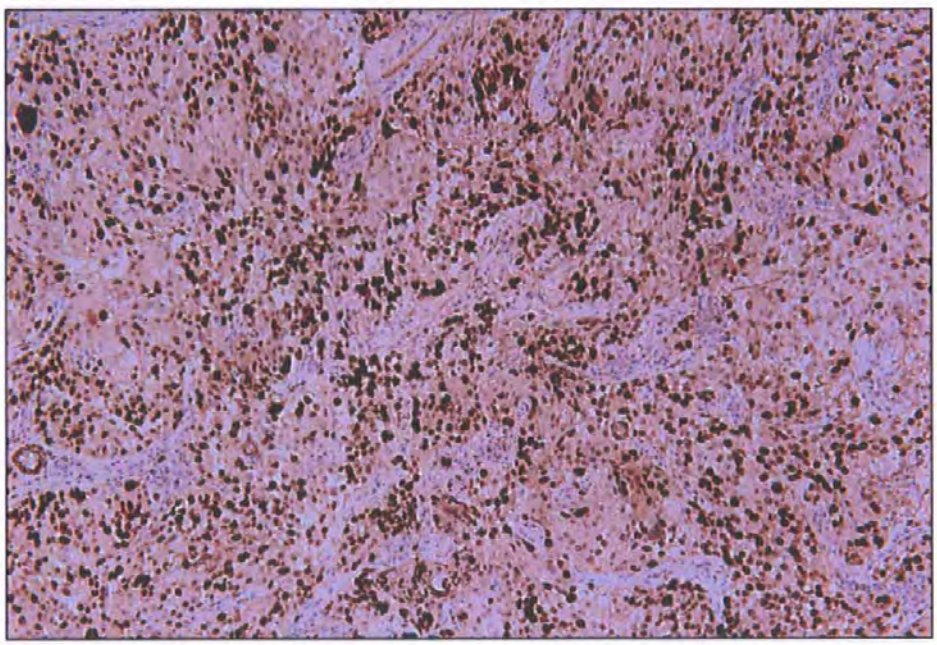


Fig. 2.6. MIB-1 positive staining:

a) Low power: area with the highest density of positively stained nuclei for MIB-1.



b) High power (X40): arrows show positive nuclear staining for MIB-1.

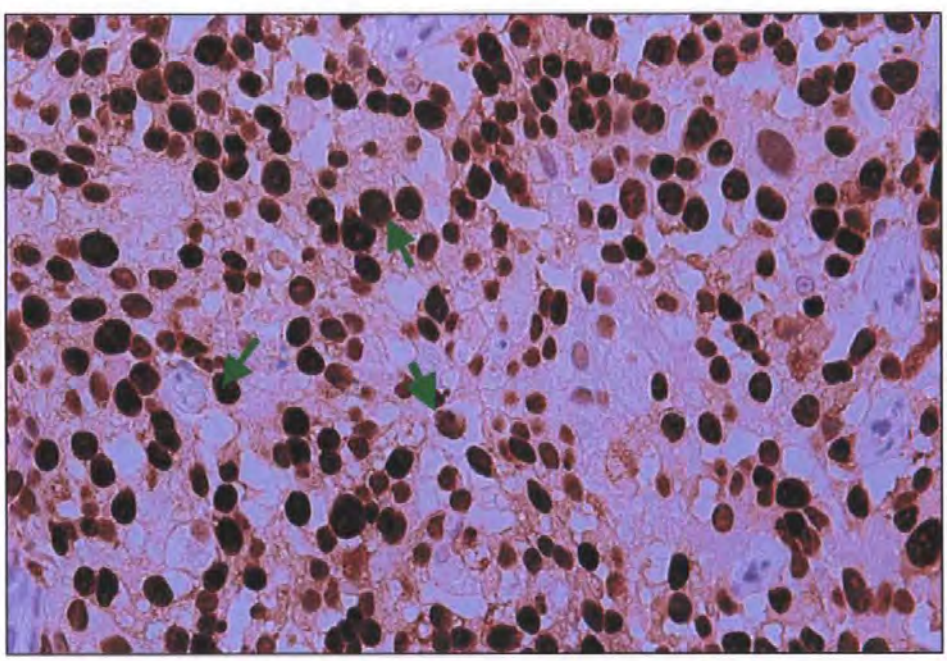
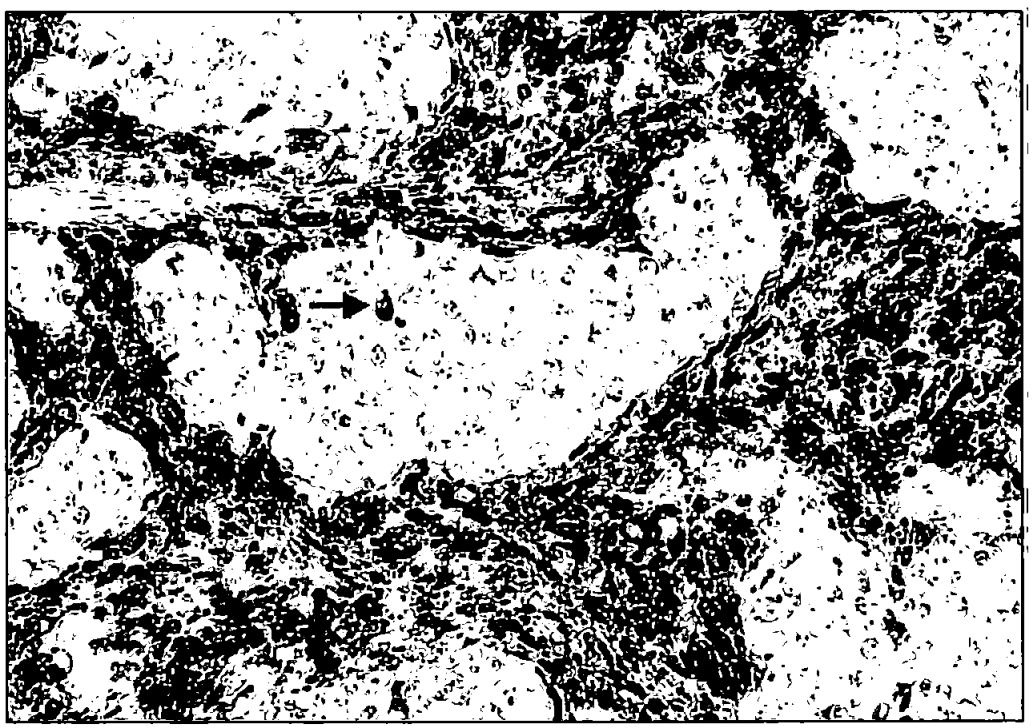


Fig. 2.7. CD68 positive cells:

a) **Low power:** This shows an area of high density of immunopositivity for the macrophage marker CD68.

b) **High power (X40):** arrows show positive immunostaining for the macrophage marker CD68.



2.3 Counting technique

Counting cell within stained sections is as follows:

(1) An eyepiece graticule (10x10 square) (Jencons-PLS, Cherrycourt way, Stanbridge Rd, Leighton Buzzard, LU7 8UA, UK) was used to aid counting; the method employed is indicated in (Fig. 2.8 a).

(2) The immunostained section was initially scanned to determine the area of high staining density at low magnification (X10).

(3) A minimum of 1000 cells nuclei were counted (stained nuclei cells + non-stained tumour cells); the number of stained nuclei within this sample was noted at high magnification (X40) (as further discussed).

(4) When calculating the number of cells within the graticule only those with nuclei positioned on the outer lines to the right and the superior lines were counted and those on the inferior and outer left lines were ignored (Fig. 2.8 b,c).

(5) The percentage of labelled nuclei was determined as indicated:

a = total number of nuclei counted (number of p53 or MIB-1 positive tumour cells + number of negative tumour cells)

b = number of labelled immunopositive cells (number of p53 or MIB-1 positive tumour cells)

Counting for macrophages (CD68):

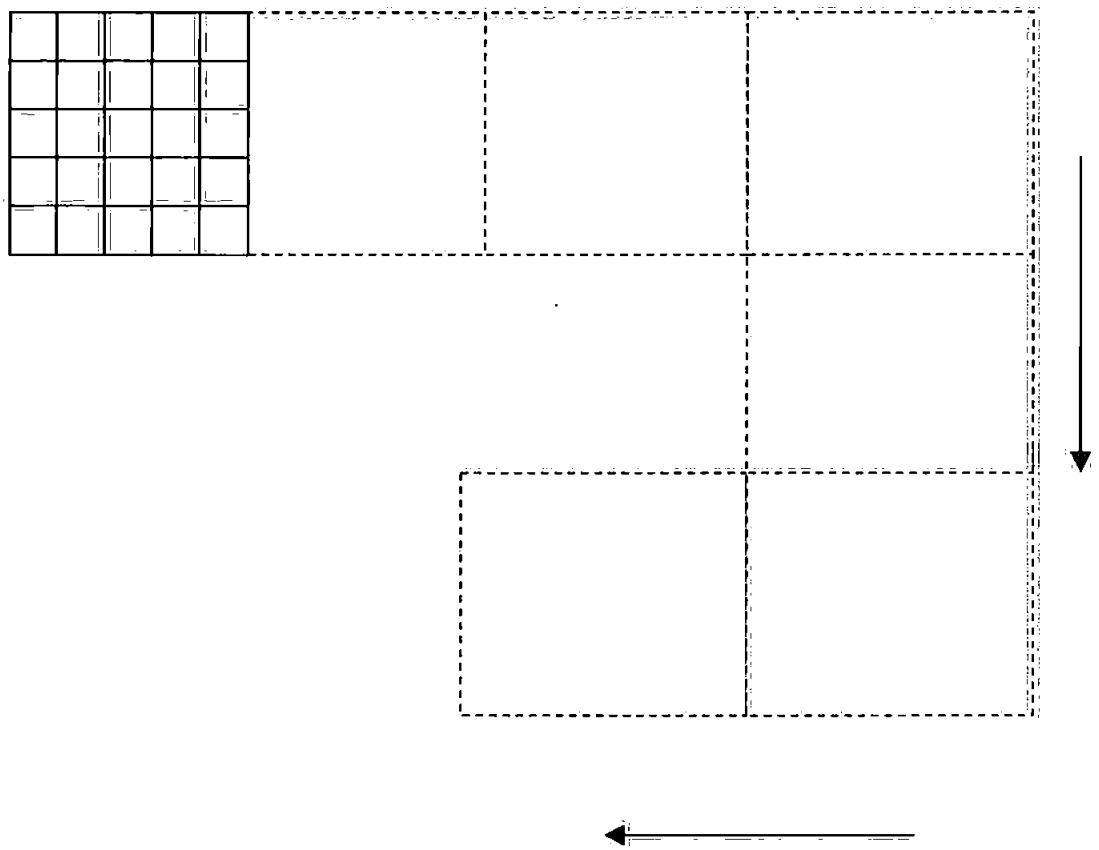
a = total number of cells counted (number of macrophages + number of tumour cells)

b = number of labelled immunopositive cells (number of CD68 positive macrophages cells)

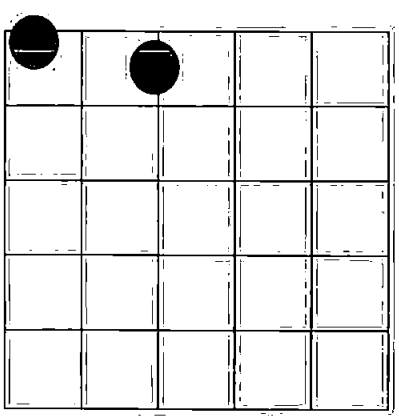
$$\text{Labelling Index [LI \%]} = \frac{\mathbf{b}}{\mathbf{a}} \times \frac{100}{1}$$

Fig. 2.8. Counting cells within stained sections:

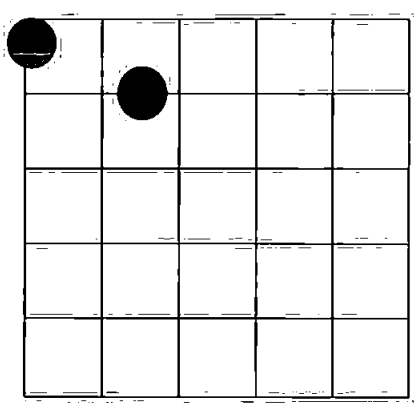
a)



b) Cells included in count



c) cells excluded in count



2.4. Statistical methods

The SPSS 10 package was used to perform the statistical analysis of the data obtained.

Univariate analysis

Categorical variables

The association between two categorical variables was assessed using the chi-squared test.

Quantitative variables

Normality of quantitative variables was checked using the Kolmogorov-Smirnov test. Variance was checked by Levine's test.

Where normality and equal variance were confirmed a t-test was used to compare means of 2 groups. Where more than 2 group means were compared Analysis of Variance (ANOVA) was used.

Where data was not normally distributed a non-parametric test was used. In such cases the Mann-Whitney test was used to compare 2 groups and the Kruskal-Wallis where there were more than 2 groups of data.

Pearson (for normally distributed data) and Spearman (for non-normally distributed data) correlation coefficients were used for correlation analysis between variables.

Regression analysis

Multiple regression analysis was used to identify variables with a significant effect on outcome. This was performed using a stepwise modal selection procedure, which eliminates insignificant variables one at a time

Survival analysis

Survival analysis was performed using the Kaplan-Meier method (Kaplan and Meier 1958).

CHAPTER THREE

RESULTS

RESULTS

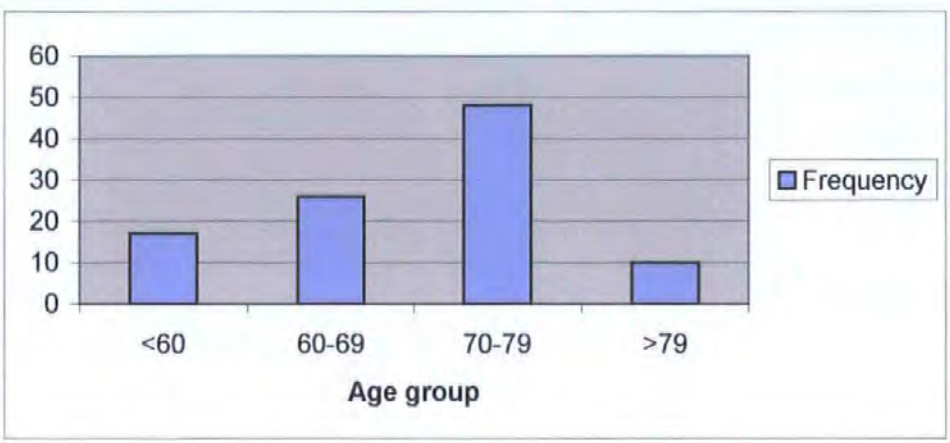
101 patients received radical external beam radiotherapy (EBRT) (range 50-60 Gy; fraction median=20) over an 8-year period. There were 85 males (84.1%) and 16 female patients (15.9%). The mean ages were 69.5 years for men and 68.6 years for women and the median ages were 71 years and 73 years respectively **(Table 3.1)**.

Table 3.1: Mean and median age by gender:

	Male	Female
Number	85	16
Median age (year)	71	73
Mean age (year)	69.5	68.6

Figure 3.1 shows the distribution of selected patients by age at diagnosis. The highest incidence is in the 70-79 year age range.

Figure 3.1. The distribution of studied bladder cancer patients by age at diagnosis:



3.1 Section one

In the first analysis 2 outcomes are considered:

Complete response to EBRT - Patients' who were found to have no tumour in the bladder at the 3-month cystoscopy.

No response to EBRT - Patients with residual tumour in the bladder at the 3-month cystoscopy.

3-months after completion of radiotherapy treatment 46 patients (45.5%) had no clinical response to radiotherapy whilst 55 (54.4%) had responded. 67 patients (66.3%) were alive and 34 (33.7%) had died during the follow up period **(Table 3.1.1).**

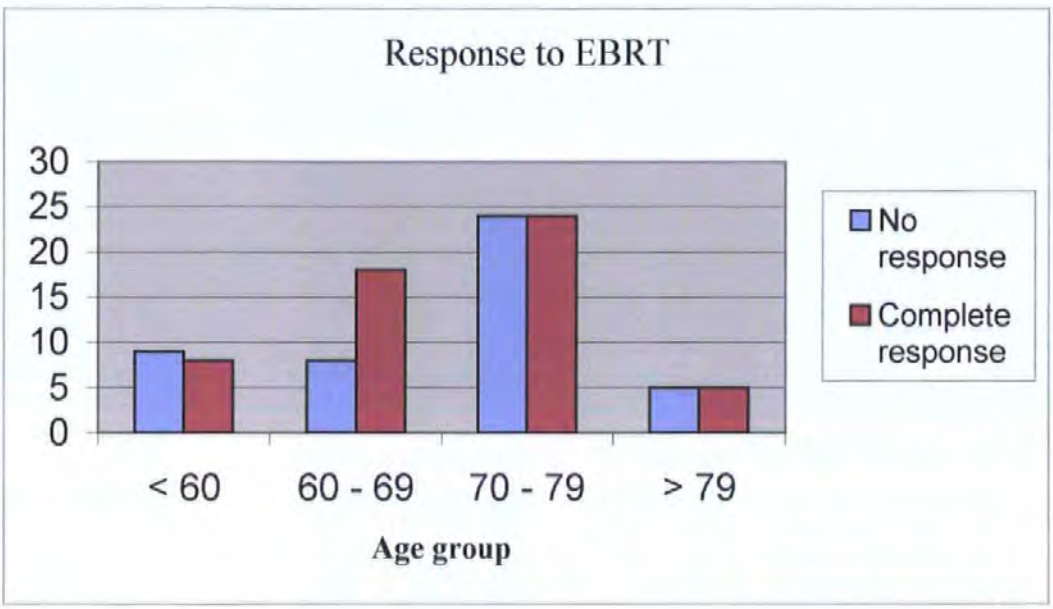
Table 3.1.1. Patient's response to EBRT and crude survival during the follow up period:

	Dead	Alive
No response to EBRT	11	35
Complete response to EBRT	23	32

3.1.1. The effects individually of age, sex, haemoglobin level and duration of treatment on the response to radiotherapy:

Figure 3.1.1, shows the age distribution of patients who responded to EBRT compared to those who did not. Patients in the age group 60-69 years appear to respond better to radiotherapy than do other age groups.

Figure 3.1.1. Distribution of Age by response to radiotherapy:



When patient's age is considered as a quantitative variable there was no significant difference between the mean ages of those patients who responded and those who did not (t-test p=0.959) **(Table 3.1.2)**.

Table 3.1.2. Mean age (SD) by response to radiotherapy:

	Mean age (SD)	p-value
No response	69.41 (9.483)	**0.959**
Complete response	69.31 (10.553)	

Sex

Females, whilst representing only a small fraction of the total (16 out of 101) showed an inferior response to radiotherapy when compared to that of male patients, and this is confirmed by Chi-Square test (p=0.048) **(Table 3.1.3)**.

Table 3.1.3. Gender of patients by response to EBRT.

	No response	Complete response	p-value
Male	35	50	**0.048**
Female	11	5	

Haemoglobin level

There was a significant difference in the median haemoglobin levels for patients who responded to EBRT and those who did not respond, higher median for response group (Mann-Whitney test, p=0.031) **(Table 3.1.4)**.

Table 3.1.4. Patients haemoglobin level by response to radiotherapy:

	No response	Complete response	p-value
Median haemoglobin g/dl (IQR)	12.75 (2.32)	13.3 (1.80)	**0.031**

*** IQR- Interquartile Range**

Duration of treatment

Duration of treatment shows no significant difference between responders and none responders (chi-squared test p=0.272) **(Table 3.1.5)**.

Table 3.1.5: Number of days by response to radiotherapy:

	No response	Complete response	p-value
Duration =<33 days	29	40	**0.272**
Duration >33 days	17	15	

3.1.2. The effects individually of tumour stage, tumour grade and site in the bladder on the response to EBRT:

Tumour stage

48 (47.5%) of the tumour were stage 2 and 53 (52.4%) were stage 3. There was no significant difference in the response to EBRT between the two stages (chi-squared test p=0.586) **(Table 3.1.6).**

Table 3.1.6. Stage of tumour by response to EBRT:

	No response	Complete response	p-value
Stage 2	20	28	
Stage 3	26	27	**0.586**

Tumour grade

11 tumours (10.8%) were grade 2 and 90 (89.1%) were grade 3. There was no significant difference between the two grades and their response to EBRT (chi-squared test p=0.753) **(Table 3.1.7).**

Table 3.1.7. Grade of tumour by response to EBRT:

	No response	Complete response	p-value
Grade 2	6	5	
Grade 3	40	50	**0.753**

Site of the tumour

Tumours were defined as occurring on the right wall (25), the left wall (30) or "other" sites (46). No significant association was found between the site of the tumour and the response to radiotherapy (chi-squared test p=0.579) **(Table 3.1.8)**.

Table 3.1.8. Site of tumour by response to EBRT:

	No response	Complete response	p-value
Right wall	11	14	**0.579**
Left wall	16	14	
"other"	19	27	

3.1.3. Tumour angiogenesis, Intratumour macrophage infiltration, p53, MIB-1, bcl-2 and the response to EBRT

Angiogenesis

46 patients did not respond to radiotherapy. The median MVD for these patients using CD31 was 4.3 (IQR 2.00) and using CD34 was 4.6 (IQR 1.47). 55 patients did respond to radiotherapy and their MVD's were 4.3 (IQR 1.70) and 4.6 (IQR 2.30) respectively. No statistically significant association was found between MVD and the response to EBRT for either method of quantifying angiogenesis using Mann-Whitney test [p=0.777 (CD31) and p= 0.686 (CD34)] **(Table 3.1.9)**.

Table 3.1.9. Median (IQR) of tumour angiogenesis by response to radiotherapy:

		Median MVD (IQR)	p-value
MVD (CD31)	No response	4.3 (2.00)	**0.777**
	Complete response	4.3 (1.70)	
MVD (CD34)	No response	4.6 (1.47)	**0.686**
	Complete response	4.6 (2.30)	

Intratumour macrophages

Macrophage infiltration (CD68) was detected in all the specimens. The CD68 labelling index (LI) ranged from 23.8% to 67.2% (mean 41.29%, SD 10.38) for none responders and 20% to 65.4% (mean 43.38, SD 11.00) for patients who did respond to EBRT. The mean CD68 labelling index showed no significant difference between patients who did not respond to EBRT and those who did respond to EBRT (t-test p= 0.331) **(Table 3.1.10)**.

Table 3.1.10. Mean (SD) of CD68 (LI) infiltrations by response to radiotherapy:

	Mean CD68 (SD)	p-value
No response	41.29 (10.38)	**0.331**
Complete response	43.38 (11.00)	

p53 expression

Nuclear p53 protein was detected in 90 tumours. 11 of the tumours stained negative for p53. The p53 labelling index (p53 LI) ranged from 0% to 99% (median 64.0, IQR 29.07) for the no response group of patients and from 0% to 99% (median 61.8, IQR 45.80) for tumours in the response group. The median p53 LI showed no significant difference between tumours that responded to EBRT and those, which did not. Using a Mann-Whitney test (p=0.230) **(Table 3.1.11)**.

Table 3.1.11. Median (IQR) of p53 (LI) infiltrations by response to radiotherapy:

EBRT	Median p53 LI (IQR)	p-value
No response	64.0 (29.07)	**0.230**
Complete response	61.8 (45.80)	

MIB-1

The MIB-1 LI of tumours ranged from 32.7% to 98.6% (mean 67.13, SD 16.17) for tumours, which did not respond, to radiotherapy and from 33.8% to 96% (mean 67.34, SD 15.41) for tumours, which did respond. There was no significant difference in the MIB-1 LI between the two groups (t-test p=0.945) **(Table 3.1.12)**.

Table 3.1.12. Mean (SD) of MIB-1 (LI) by response to radiotherapy:

EBRT	Mean MIB-1 LI (SD)	p-value
No response	67.13 (16.17)	**0.945**
Complete response	67.34 (15.41)	

Bcl-2

48 (47.5%) of the tumours showed bcl-2 expression and 53 (52.5%) no bcl-2 expression. The response of the two groups to EBRT is shown in the following table. No significant association was found for bcl-2 scoring and the response to EBRT (chi-squared test p=0.291) **(Table 3.1.13).**

Table 3.1.13. Response to radiotherapy by bcl-2:

	No response	Complete response	p-value
Bcl-2 +ve	25	23	**0.291**
Bcl-2 -ve	21	32	

3.1.4 Association between MVD, haemoglobin, macrophage infiltration, MIB-1, bcl-2, p53 and tumour characteristics:

MVD and stage

MVD quantified by CD31 showed no significant association with tumour stage (Mann-Whitney test p=0.469). When MVD was quantified using CD34 a significant difference was observed; MVD being lower in higher stage tumours (Mann-Whitney test p=0.050) **(Table 3.1.14).**

Table 3.1.14. Median (IQR) of MVD by stage of bladder TCC:

	Tumour stage	Median MVD (IQR)	P value
CD31	2	4.3 (2.22)	**0.469**
	3	4.3 (2.00)	
CD34	2	4.8 (2.00)	**0.050**
	3	4.3 (1.40)	

MVD and grade

There were 11 grade 2 tumours and 90 grade 3 tumours. The median MVD for grade 2 tumours was 4.00 (IQR 2.00) "for grade tumours" using CD31 and 3.60 (IQR 2.70) using CD34. The respective values for grade 3 tumours were 4.3 (IQR 2.00) and 4.60 (IQR 1.78). There was no significant difference in the MVD for grade 2 (Mann-Whitney test, p=0.525) and grade 3 tumours (Mann-Whitney test, p=0.252) when stained by either antibody **(Table 3.1.15).**

Table 3.1.15. Median (IQR) of MVD by grade of bladder TCC:

	Tumour grade	Median MVD (IQR)	p value
CD31	2	4.00 (2.00)	**0.525**
	3	4.3 (2.00)	
CD34	2	3.60 (2.70)	**0.252**
	3	4.60 (1.78)	

MVD and tumour site

25 tumours were on the right wall of the bladder. The median MVD for these tumours was 4.3 (IQR 2.30) using CD31 and 5.00 (IQR 1.95) using CD34. 30 tumours were on the left wall and the median MVD of these was 4.3 (IQR 1.78) using CD31 and 4.6 (IQR 1.78) using CD34. The remaining 46 tumours were found at "other" sites in the bladder and the median MVD of these was 4.15 (IQR 2.00) using CD31 and 4.3 (IQR 1.78) using CD34. There was no significant association between MVD (when quantified by either CD31 or CD34) and the site of the tumour (Kruskall-Wallis, p=0.398 and p=0.410) **(Table 3.1.16).**

Table 3.1.16. Median (IQR) of MVD by site of bladder TCC:

	Site	Median MVD (IQR)	p value
CD31	Right wall	4.3 (2.30)	**0.398**
	Left wall	4.3 (1.78)	
	"Other"	4.15 (2.00)	
CD34	Right wall	5.0 (1.95)	**0.410**
	Left wall	4.6 (1.78)	
	"Other"	4.3 (1.78)	

Haemoglobin and stage

There was no significant difference between haemoglobin level in the different tumour stages (Mann-Whitney test, p=0.388) **(Table 3.1.17).**

Table 3.1.17. Haemoglobin and stage of TCC:

	Median HB (IQR)	**p-value**
Stage 2	13.1 (2.30)	**0.388**
Stage 3	12.8 (2.20)	

Haemoglobin and grade

There was no significant difference between haemoglobin levels in the different tumour grades (Mann-Whitney test, p=0.905) **(Table 3.1.18).**

Table 3.1.18. Haemoglobin and grade of TCC:

	Median HB (IQR)	**p-value**
Grade 2	12.70 (3.00)	**0.905**
Grade 3	13.00 (2.10)	

Haemoglobin and tumour site

There was no significant association between haemoglobin level and site of the tumour (Kruskall-Wallis, p=0.966) **(Table 3.1.19).**

Table 3.1.19. Haemoglobin and site of TCC:

	Median HB (IQR)	**p-value**
Right wall	13.00 (2.90)	**0.966**
Left wall	12.95 (1.86)	
"Other"	13.05 (2.38)	

CD 68 LI and stage

There was no significant difference between macrophage infiltrations in the different tumour stages (t-test p=0.23) **(Table 3.1.20).**

Table 3.1.20. CD68 and stage of TCC:

	Mean CD 68 LI (SD)	**p-value**
Stage 2	43.78 (10.44)	**0.23**
Stage 3	41.20 (10.91)	

CD 68 LI and tumour grade

There was no significant difference between macrophage infiltrations in the different tumour grades (t-test p=0.75) **(Table 3.1.21)**.

Table 3.1.21. CD68 and grade of TCC:

	Mean CD 68 LI (SD)	**p-value**
Grade 2	43.41 (11.36)	**0.75**
Grade 3	42.31 (10.70)	

CD 68 LI and tumour site

There was no significant association between macrophage infiltration and site of the tumour (AVOVA p=0.93) **(Table 3.1.22).**

Table 3.1.22. CD68 and site of TCC:

	Mean CD68 LI (SD)	**p-value**
Right wall	41.88 (11.03)	**0.93**
Left wall	42.24 (10.86)	
"Other"	42.84 (10.67)	

Bcl-2 and tumour stage

26 of the 48 stage 2 tumours expressed bcl-2 and 22 of the 53 stage 3 tumours showed bcl-2 expression. No significant association was found between bcl-2 expression and tumour stage (chi-square test p= 0.203) **(Table 3.1.23)**.

Table 3.1.23. Association between bcl-2 and stage of bladder TCC:

	bcl-2 +ve	bcl-2 -ve	P value
Stage 2	26	22	**0.203**
Stage 3	22	31	

Bcl-2 and tumour grade

11 tumours were grade 2 and of these 8 showed expression of bcl-2. 90 tumours were grade 3 and of these 40 showed expression of bcl-2. No significant association was found between bcl-2 expression and tumour grade (Chi-square test p=0.076) **(Table 3.1.24).**

Table 3.1.24. Association between bcl-2 and grade of bladder TCC:

	bcl-2 +ve	bcl-2 -ve	P value
Grade 2	8	3	**0.076**
Grade 3	40	50	

Bcl-2 and tumour site

12 out of 25 tumours on the right wall; 14 out of 30 tumours on the left wall and 22 tumours found at "other" sites in the bladder showed bcl-2 expression. There was no significant association between bcl-2 expression and tumour site in the bladder (chi-square test p=0.994) **(Table 3.1.25).**

Table 3.1.25. Association between bcl-2 and site of bladder TCC:

	bcl-2 +ve	bcl-2 -ve	P value
Right wall	12	13	**0.994**
Left wall	14	16	
"other site"	22	24	

MIB-1 and tumour stage

The mean MIB-1 LI of the 48 stage 2 tumours was 67.99 (SD 14.86) and that for the 53 grade 3 tumours was 66.57 (SD 16.50). There was no significant difference between the two groups (t-test p=0.653) **(Table 3.1.26).**

Table 3.1.26. Association between MIB-1 (L I) and stage of bladder TCC:

	Mean MIB-1 LI (SD)	P value
Stage 2	67.99 (14.86)	**0.653**
Stage 3	66.57 (16.50)	

MIB-1 and tumour grade

The mean MIB-1 LI for the 11 grade 2 tumours was 65.80 (SD 21.83) and the mean MIB-1 LI for the 90 grade 3 tumours was 67.42 (SD 14.92). There was no significant association between MIB-1 and tumour grade (t-test p=0.748) **(Table 3.1.27)**.

Table 3.1.27. Association between MIB-1 and grade of bladder TCC:

	Mean MIB-1 LI (SD)	P value
Grade 2	65.80 (21.83)	**0.748**
Grade 3	67.42 (14.92)	

MIB-1 and tumour site

The mean MIB-1 LI for tumours on the right wall was 62.96 (SD 14.73), for those on the left wall 69.26 (SD 15.39) and for those at "other" sites 68.26 (SD 16.25). No significant association was found between MIB-1 LI and tumour site (ANOVA p=0.28) **(Table 3.1.28)**.

Table 3.1.28. Association between MIB-1 and site of bladder TCC:

	Mean MIB-1 LI (SD)	p-value
Right wall	62.96 (14.73)	**0.28**
Left wall	69.26 (15.39)	
"other" site	68.26 (16.25)	

p53 and tumour stage

The median p53 LI of stage 2 tumours was 64.4 (IQR 41.15) and that for stage 3 tumours was 59.4 (IQR 42.55). There was no significant difference between the two groups (Mann-Whitney test, p= 0.522) **(Table 3.1.29).**

Table 3.1.29. Association between p53 (LI) and stage of bladder TCC:

	Median p53 LI (IQR)	**p-value**
Stage 2	64.4 (41.15)	**0.522**
Stage 3	59.4 (42.55)	

p53 and tumour grade

The median p53 LI for grade 2 tumours was 70.00 (IQR 53.70) and that for grade 3 tumours was 63.40 (IQR 39.18). There was no significant difference in the p53 staining for grade 2 and grade 3 tumours (Mann-Whitney test, p=0.616) **(Table 3.1.30)**.

Table 3.1.30. Association between p53 (LI) and grade of bladder TCC:

	Median p53 LI (IQR)	**p-value**
Grade 2	70.00 (53.70)	**0.616**
Grade 3	63.40 (39.18)	

p53 and tumour site

The median p53 LI of tumours on the right wall of the bladder was 43.9 (IQR 59.35), on the left wall 66.8 (IQR 23.97) and for "other" sites in the bladder it was 58.05 (IQR 39.95). There was no significant difference in p53 LI according to the site of the tumour (Kruskall-Wallis, p=0.116) **(Table 3.1.31).**

Table 3.1.31. Association between p53 (LI) and site of bladder TCC:

	Median p53 LI (IQR)	**P value**
Right wall	43.9 (59.35)	**0.116**
Left wall	66.8 (23.97)	
"other" site	58.05 (39.95)	

3.1.5 Association between other variables:

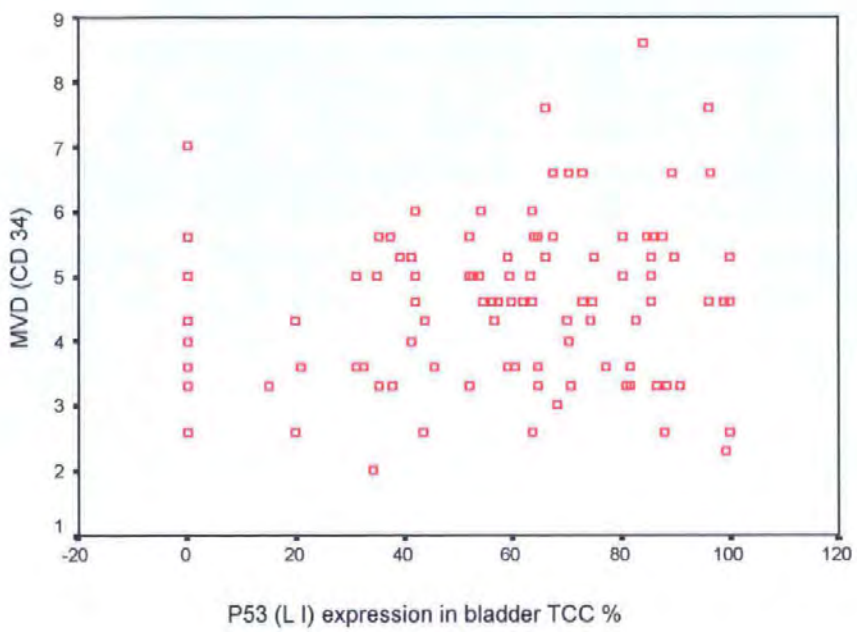
p53 and MVD (CD31, CD34)

p53 is not correlated (Spearman correlation) with CD31 (r=0.187, p=0.062) and CD34 (r=0.167, p=0.096) **(Table 3.1.32, figure 3.1.2).**

Table 3.1.32. Correlation between p53 (LI) and MVD in bladder TCC:

		Tumour angiogenesis CD31	Tumour angiogenesis CD34
p53 (LI) expression in bladder TCC %	Correlation coefficient	0.187	0.167
	Sig. (2-tailed)	0.062	0.096
	N	101	101

Figure 3.1.2. Scatter plot of p53 Vs MVD (CD34):



Scatter plot of p53 Vs MVD (CD31) see appendix (page 203).

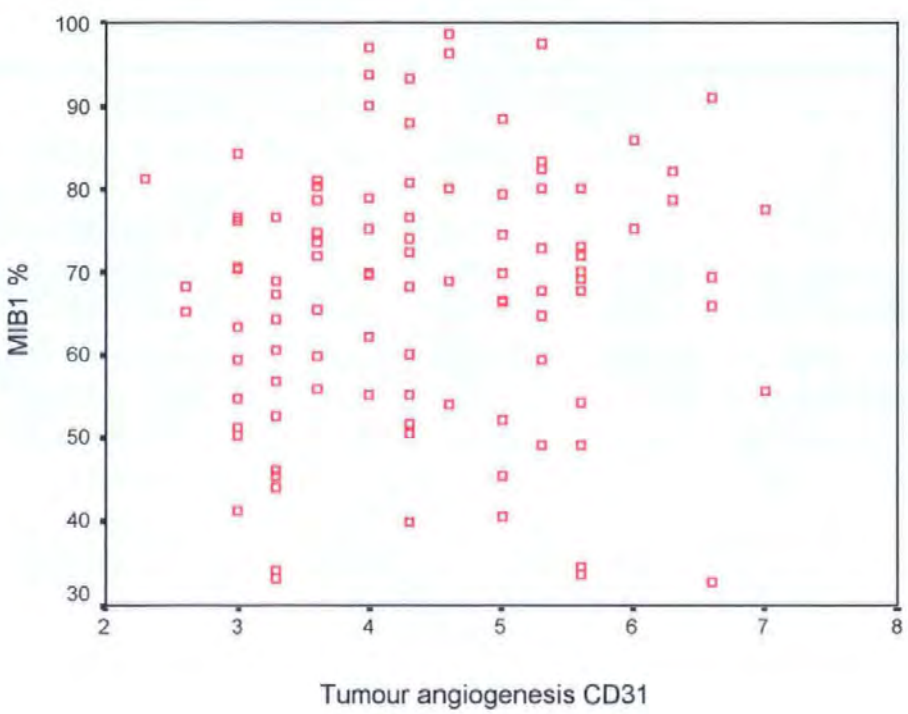
MIB-1 and MVD (CD31, CD34)

MIB-1 is not correlated (Spearman correlation) with CD31 (r=0.134, p=0.153) and CD34 (r=0.114, p=0.254) **(Table 3.1.33, figure 3.1.3).**

Table 3.1.33. Correlation between MIB-1 (LI) and MVD in bladder TCC:

		Tumour angiogenesis CD31	Tumour angiogenesis CD34
Proliferation of tumour cells (MIB1) %	Correlation coefficient	0.134	0.114
	Sig. (2-tailed)	0.153	0.254
	N	101	101

Figure 3.1.3. Scatter plot of MIB-1 Vs MVD (CD31):



Scatter plot of MIB-1 Vs MVD (CD34) see appendix (page 202).

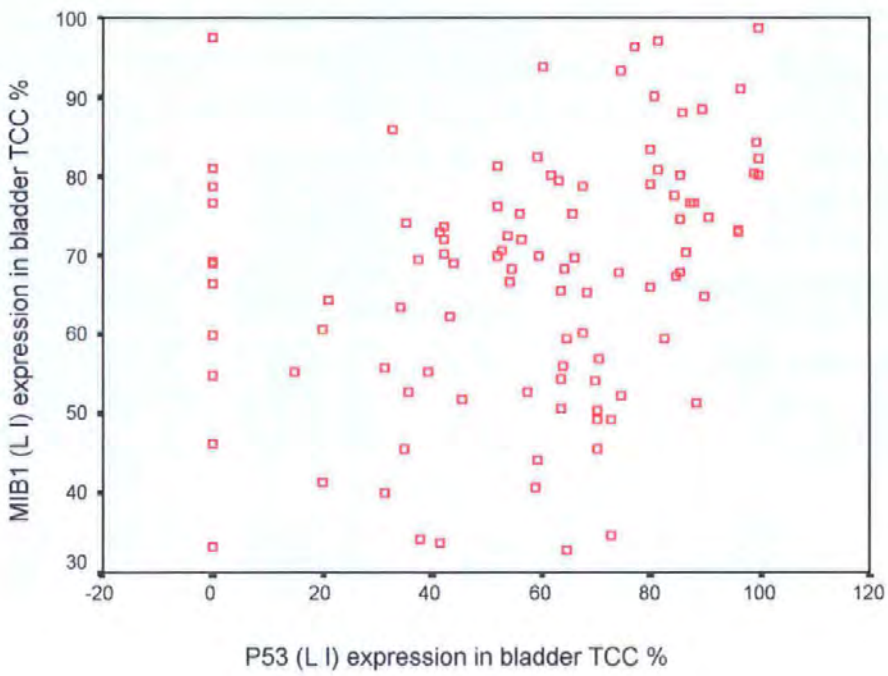
p53 and MIB-1

There was a significant Spearman correlation (positive) between p53 LI expression and MIB-1 LI (r=0.332, p=0.001) **(Table 3.1.34, figure 3.1.4)**.

Table 3.1.34. Correlation between p53 (LI) and MIB-1 in bladder TCC:

		Proliferation of tumour cells MIB1 %
p53 (LI) expression in bladder TCC %	Correlation coefficient	0.332
	Sig. (2-tailed)	0.001
	N	101

Figure 3.1.4. Scatter plot of p53 Vs MIB-1:



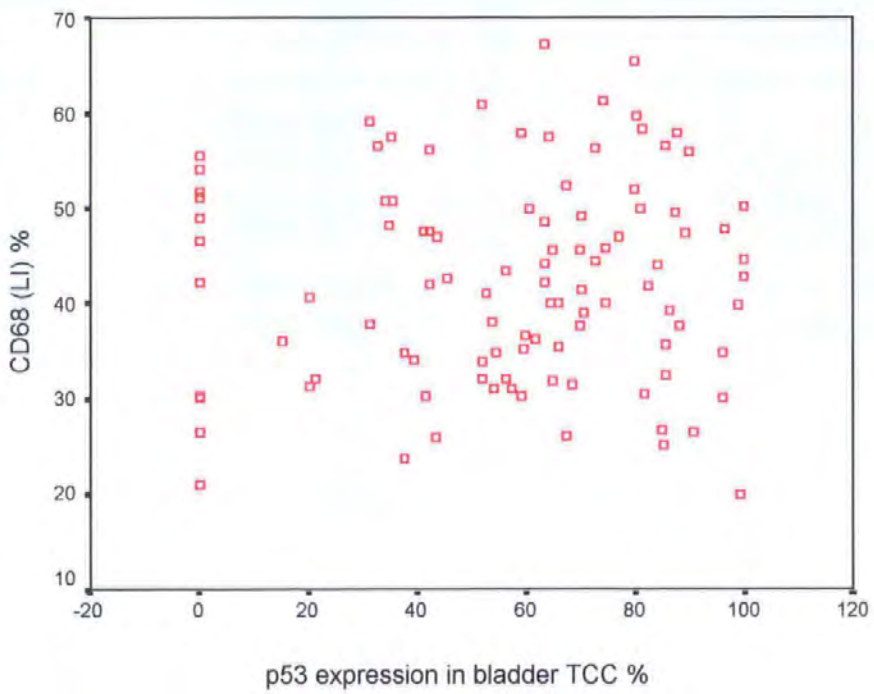
p53 and CD68

There was no significant positive Spearman correlation between p53 and CD68 (r=0.036, p=0.719) **(Table 3.1.35, figure 3.1.5)**.

Table 3.1.35. Correlation between p53 and CD68 (LI) in bladder TCC:

		Tumour macrophage CD68
p53 (LI) expression in bladder TCC %	Correlation coefficient	0.036
	Sig. (2-tailed)	0.719
	N	101

Figure 3.1.5. Scatter plot of p53 Vs CD68:



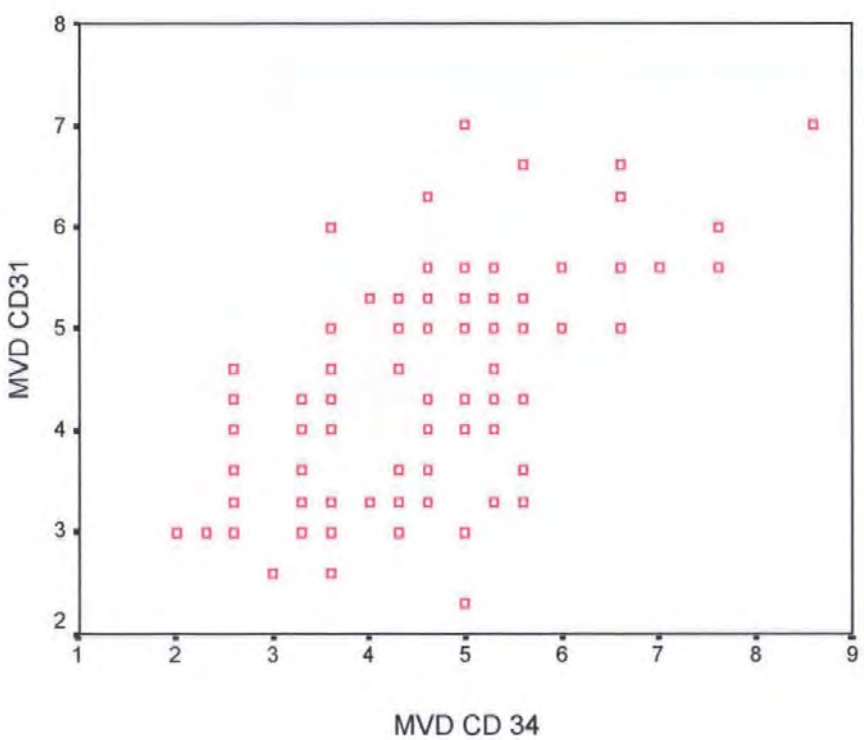
CD31 and CD34

A significant positive Spearman correlation is found between CD31 and CD34 ($r=0.589$, $p<0.001$) **(Table 3.1.36, figure 3.1.6)**.

Table 3.1.36. Correlation between CD31 and CD34 in bladder TCC:

		Tumour angiogenesis CD34
Tumour angiogenesis CD31	Correlation coefficient	0.589
	Sig. (2-tailed)	0.000
	N	101

Figure 3.1.6. Scatter plot of CD31 Vs CD34:



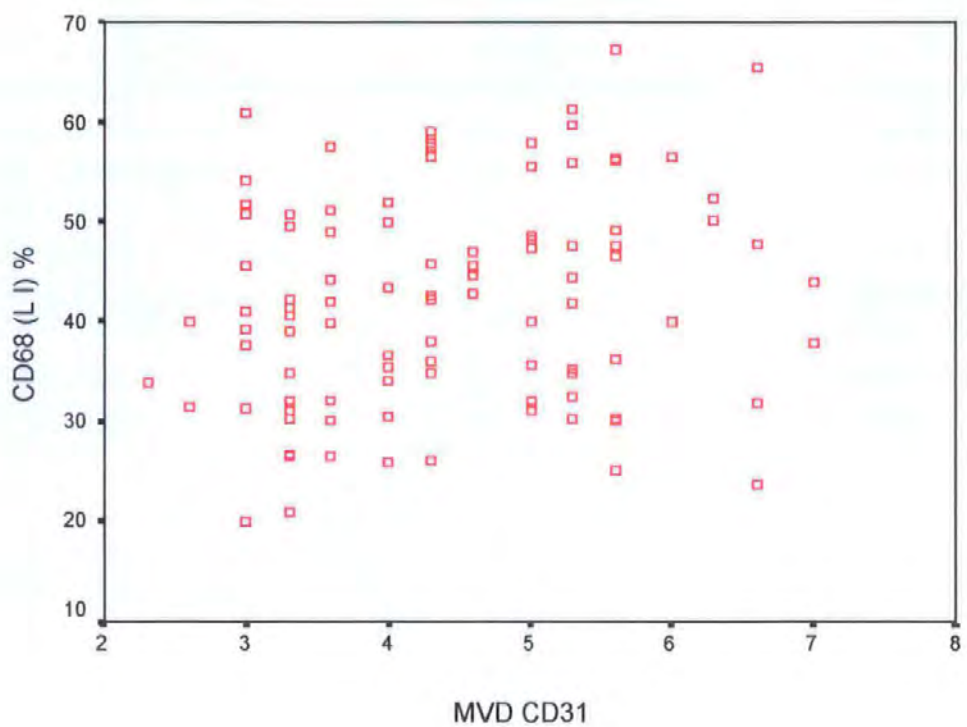
CD31 and CD68

There was no significant positive Spearman correlation between CD31 and CD68 (r=0.192, p=0.054) **(Table 3.1.37, figure 3.1.7)**.

Table 3.1.37. Correlation between CD68 (LI) and MVD in bladder TCC:

		Tumour macrophage CD68
Tumour angiogenesis CD31	Correlation coefficient	0.192
	Sig. (2-tailed)	0.054
	N	101

Figure 3.1.7. Scatter plot of CD68 Vs MVD (CD31):



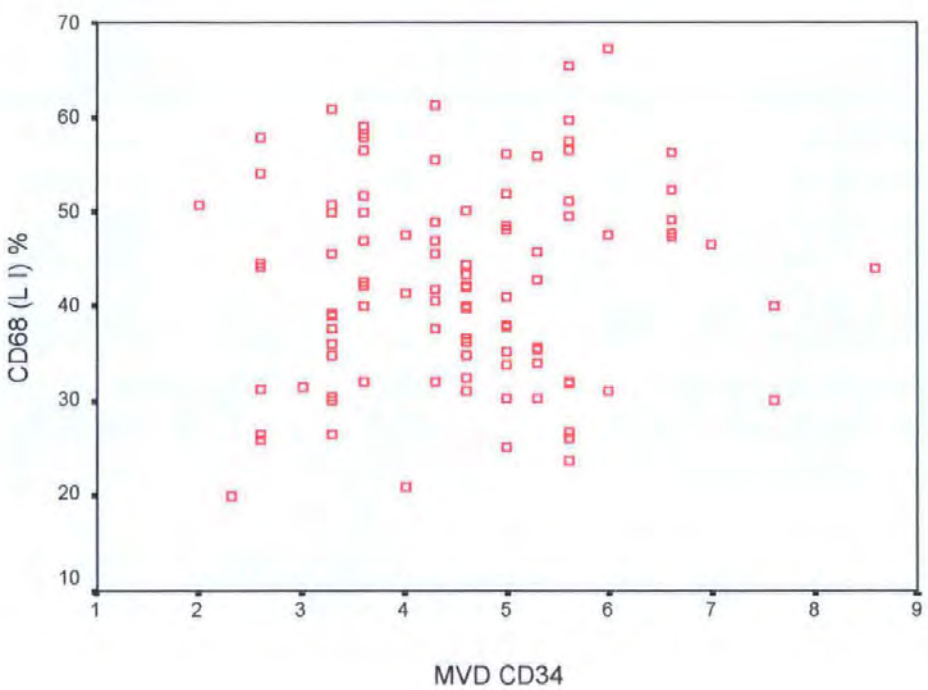
CD34 and CD68

There was no significant Spearman correlation between CD34 and CD68 (r=0.078, p=0.436) **(Table 3.1.38, figure 3.1.8)**.

Table 3.1.38. Correlation between CD68 (LI) and MVD in bladder TCC:

		Tumour angiogenesis CD34
Tumour macrophage CD68	Correlation coefficient	0.078
	Sig. (2-tailed)	0.436
	N	101

Figure 3.1.8. Scatter plot of CD68 Vs MVD (CD34):



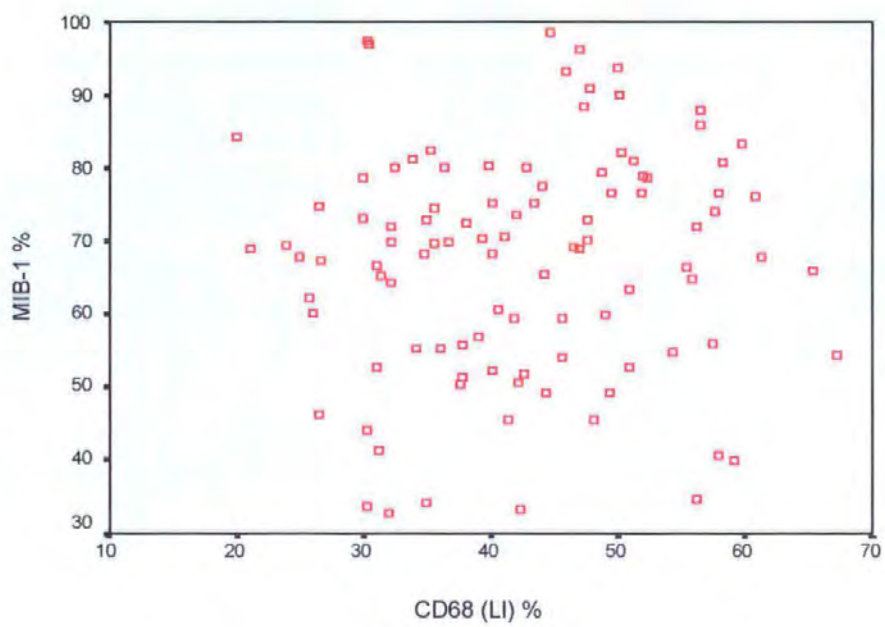
MIB-1 and CD68

There was no significant Pearson correlation between MIB-1 and CD68 (r=0.072, p=0.475) **(Table 3.1.39, figure 3.1.9)**.

Table 3.1.39. Correlation between MIB-1 and CD68 (LI) in bladder TCC:

		Tumour macrophage CD68
Proliferation of tumour cells MIB1 %	Correlation coefficient	0.072
	Sig. (2-tailed)	0.475
	N	101

Figure 3.1.9. Scatter plot of CD68 Vs MIB-1:



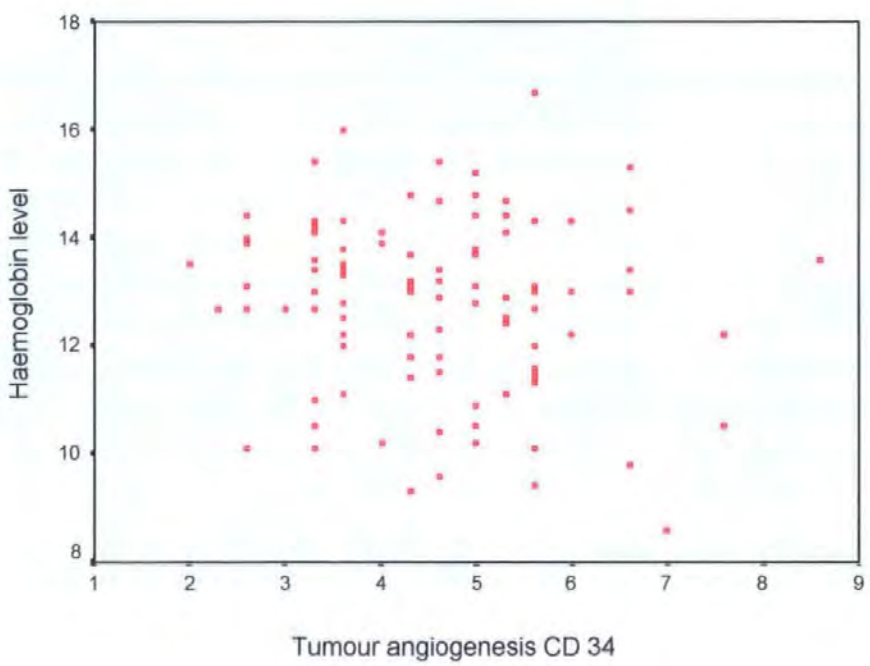
Haemoglobin and MVD (CD31&CD34)

Haemoglobin level is not correlated (Spearman correlation) with CD31 (r=0.020, p=0.845) and CD34 (r= -0.132, p=0.187) **(Table 3.1.40, figure 3.1.10).**

Table 3.1.40. Correlation between haemoglobin level and MVD in bladder TCC:

		Tumour angiogenesis CD31	Tumour angiogenesis CD34
Haemoglobin level	Correlation coefficient	0.020	-0.132
	Sig. (2-tailed)	0.845	0.187
	N	101	101

Figure 3.1.10. Scatter plot of haemoglobin level Vs MVD (CD34):



Scatter plot of haemoglobin level Vs MVD (CD31) see appendix.

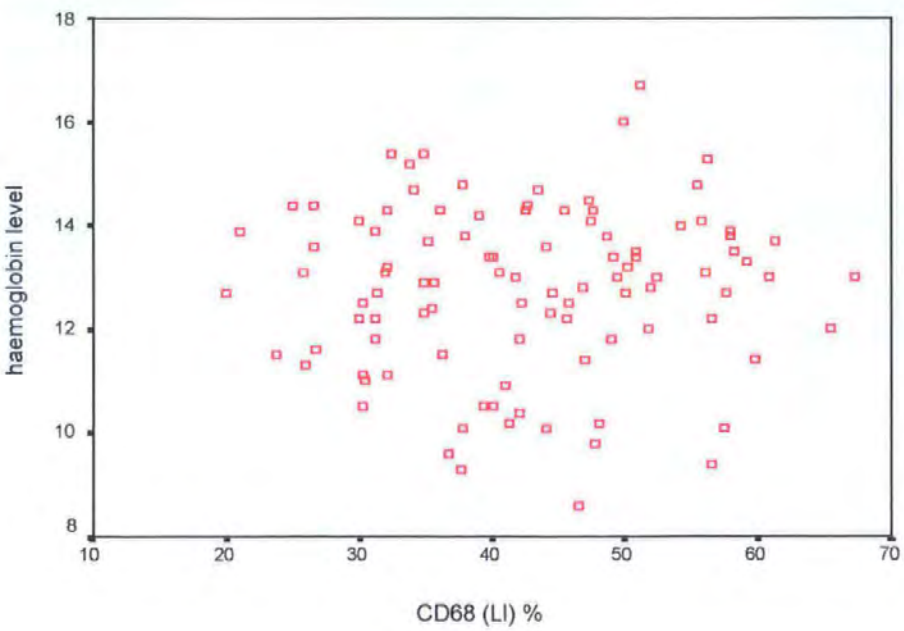
Haemoglobin and CD68

There was no significant Spearman correlation between haemoglobin level and CD68 (r=0.051, p=0.612) **(Table 3.1.41, figure 3.1.11)**.

Table 3.1.41. Correlation between haemoglobin and CD68 (LI) in bladder TCC:

		Tumour macrophage CD68
Haemoglobin level	Correlation coefficient	0.051
	Sig. (2-tailed)	0.612
	N	101

Figure 3.1.11. Scatter plot of haemoglobin level Vs CD68:



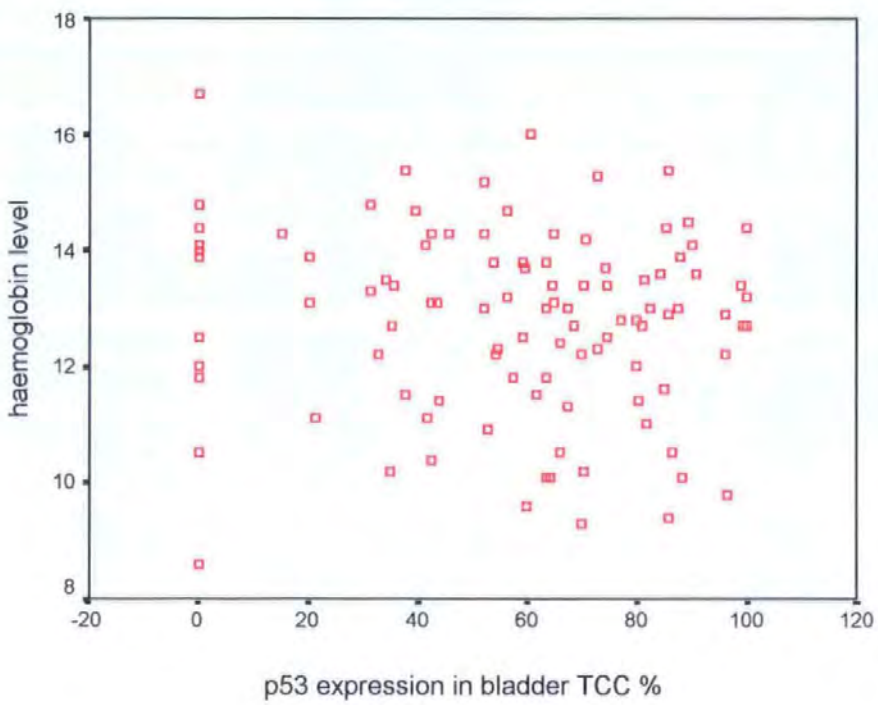
Haemoglobin and p53

Haemoglobin level is not correlated (Spearman correlation) with p53 (r= -0.108, p=0.281) **(Table 3.1.42, figure 3.1.12).**

Table 3.1.42. Correlation between haemoglobin and p53 (LI) in bladder TCC:

		p53 (LI) expression in bladder TCC %
Haemoglobin level	Correlation coefficient	-0.108
	Sig. (2-tailed)	0.281
	N	101

Figure 3.1.12. Scatter plot of haemoglobin level Vs p53:



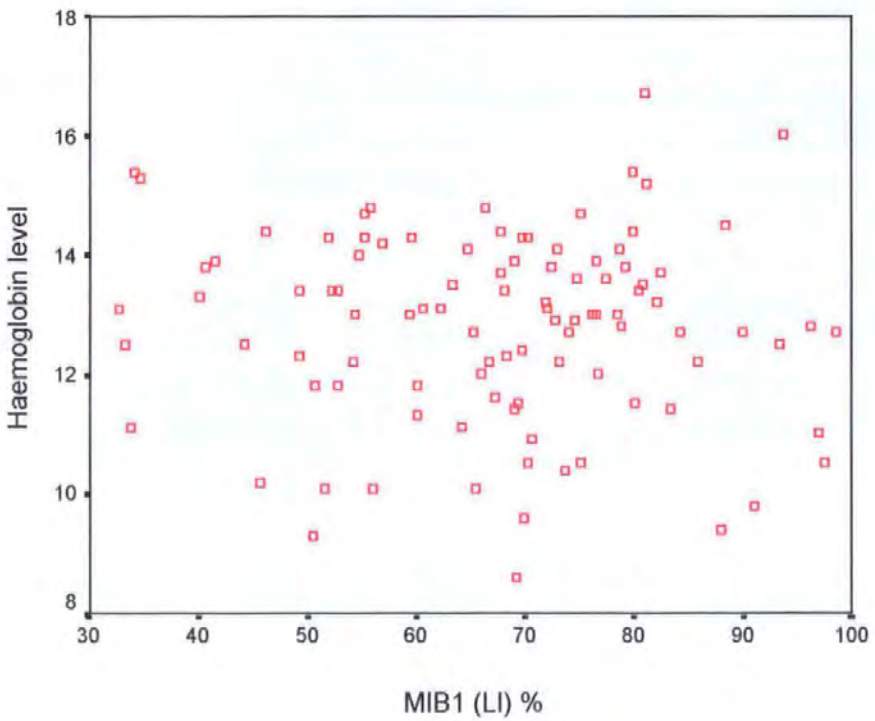
Haemoglobin and MIB-1

There was no significant Spearman correlation between haemoglobin level and MIB-1 (r= -0.011, p=0.913) **(Table 3.1.43, figure 3.1.13)**.

Table 3.1.43. Correlation between haemoglobin and MIB-1 (LI) in bladder TCC:

		Proliferation of tumour cells MIB1 %
Haemoglobin level	Correlation coefficient	-0.011
	Sig. (2-tailed)	0.913
	N	101

Figure 3.1.13. Scatter plot of haemoglobin level Vs MIB-1:



Bcl-2 and MVD (CD31)

There was no significant Spearman correlation between bcl-2 and CD31 (r=0.039, p=0.697) **(Table 3.1.44).**

Table 3.1.44. Correlation between Bcl-2 and MVD (CD31) in bladder TCC:

		Tumour angiogenesis CD31
Bcl-2	Correlation coefficient	0.039
	Sig. (2-tailed)	0.697
	N	101

Bcl-2 and MVD (CD34)

There was no significant Spearman correlation between bcl-2 and CD34 (r= -0.056, p=0.580) **(Table 3.1.45)**

Table 3.1.45. Correlation between Bcl-2 and MVD (CD34) in bladder TCC:

		Tumour angiogenesis CD34
Bcl-2	Correlation coefficient	-0.056
	Sig. (2-tailed)	0.580
	N	101

Bcl-2 and CD68

There was no significant Spearman correlation between bcl-2 and CD68 (r= 0.027, p=0.792) **(Table 3.1.46)**

Table 3.1.46. Correlation between Bcl-2 and CD68 in bladder TCC:

		Intratumour macrophage CD68
Bcl-2	Correlation coefficient	0.027
	Sig. (2-tailed)	0.792
	N	101

Bcl-2 and p53

There was no significant Spearman correlation between bcl-2 and p53 (r= -0.129, p=0.199) **(Table 3.1.47)**

Table 3.1.47. Correlation between Bcl-2 and p53 in bladder TCC:

		p53 expression in bladder TCC %
Bcl-2	Correlation coefficient	-0.129
	Sig. (2-tailed)	0.199
	N	101

Bcl-2 and MIB-1

There was no significant Spearman correlation between bcl-2 and MIB-1 (r= -0.066, p=0.514) **(Table 3.1.48)**

Table 3.1.48. Correlation between Bcl-2 and MIB-1 in bladder TCC:

		Proliferation of tumour cells MIB-1
Bcl-2	Correlation coefficient	-0.066
	Sig. (2-tailed)	0.514
	N	101

Bcl-2 and haemoglobin level

There was a marginally significant Spearman correlation between bcl-2 and haemoglobin level (r= 0.197, p=0.049) **(Table 3.1.49)**

Table 3.1.49. Correlation between Bcl-2 and haemoglobin level in bladder TCC:

		Haemoglobin level
Bcl-2	Correlation coefficient	0.197
	Sig. (2-tailed)	0.049
	N	101

3.1.6 Multivariate analysis of the effects of the variables with respect to the response to radiotherapy:

A multivariate logistic regression was fitted to the data with response to radiotherapy as the dependant variable and the other variables as independent. Because of the correlation between predictors, CD34 and MIB1 were not used in the logistic regression model to avoid multicollinearity.

The backward stepwise logistic regression shows that only sex seemed to be marginally significant, for instance the odds of response for a male patient is 3 times the odds of response for female adjusting for other variables. **(Table 3.1.50).**

Table 3.1.50. Predictors for response to radiotherapy:

	B	Sig.	Odds ratio	95.0% C.I. for OR	
				Lower	Upper
[a] **HB**	0.259	0.060	1.296	0.989	1.699
Sex	-1.177	0.051	0.308	0.095	1.006
p53	-0.013	0.091	0.987	0.972	1.002
Constant	-1.001	0.611	0.368		

[a] Variable(s) entered on step 1: Age, Grade, HB, Site, Stage, CD31, CD68, Days, Sex, Radioth. Dose, Bcl-2 and p53.

3.2 Section two

In this second analysis the two outcomes are as follows: -

Enduring complete response to EBRT – patients in whom bladder tumour was not found at any follow up cystoscopy.

Failed treatment – patients who at any follow up cystoscopy were found to have bladder tumour.

The two outcomes were used to analyse the data as above.

In 42 (41.6%) patients there was a complete response to EBRT whilst 59 (58.4%) were considered treatment failures. During the 8-year follow up period 67 patients (66.3%) were alive and 34 (33.7%) had died **(Table 3.2.1).**

Table 3.2.1. Patient's response to EBRT:

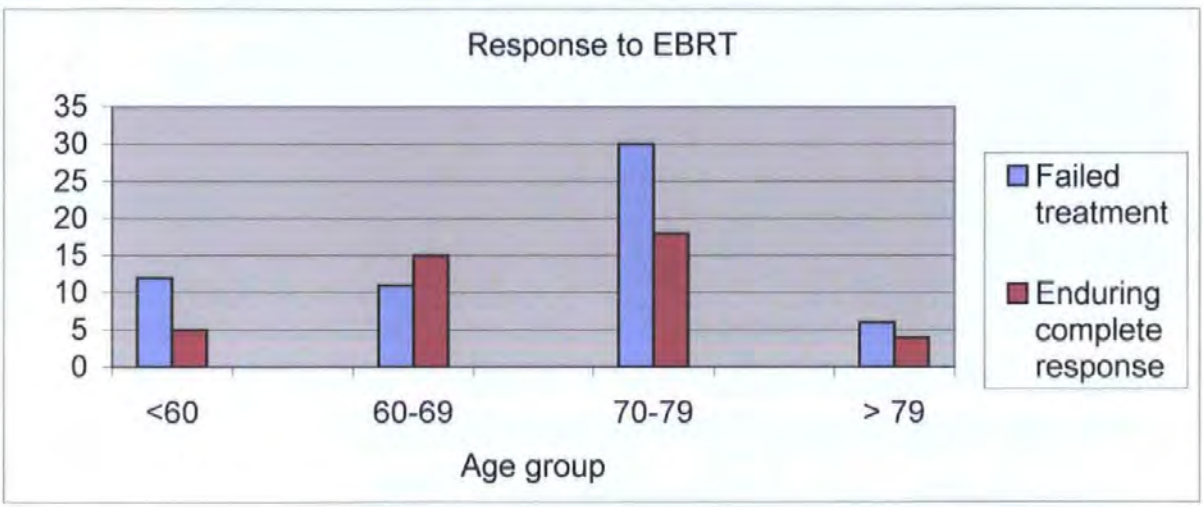
EBRT	Dead	Alive
Failed treatment	16	43
Enduring complete response to	18	24

3.2.1. The effects individually of age, sex, haemoglobin level and duration of treatment on the response to radiotherapy:

Age

Figure 3.2.1, shows the age distribution of patients who responded to EBRT compared to those who did not. Patients in the age group 60-69 years appear to respond better to radiotherapy than do other age groups.

Figure 3.2.1. Age distribution by response to EBRT:



When patient's age is considered as a quantitative variable there was no significant difference between the mean ages of those patients who responded and those who did not (t-test p=0.477) **(Table 3.2.2)**.

Table 3.2.2. Mean age (SD) by response to radiotherapy:

EBRT	Mean age (SD)	p-value
Failed treatment	69.12 (10.41)	**0.477**
Enduring complete response	69.69 (9.58)	

Sex

In this study the ratio male to female is about 5:1. Females (15.8%), whilst representing only a small fraction of the total (16 out of 101) showed an inferior response (12.5%) to radiotherapy when compared to that of male patients (47.1%), and this is confirmed by Chi-Square test (p=0.022) **(Table 3.2.3).**

Table 3.2.3. Gender of patients by response to EBRT:

SEX	Failed treatment	Enduring complete response	p-value
Male	45	40	**0.022**
Female	14	2	

Haemoglobin level

There was no significant difference in the mean haemoglobin levels for patients who responded to EBRT and those who did not respond (Mann-Whitney test, p=0.107) **(Table 3.2.4).**

Table 3.2.4. Patients haemoglobin level by response to radiotherapy:

	Failed treatment	Enduring complete response	p-value
Median haemoglobin g/dl (IQR)	12.80 (2.40)	13.20 (1.55)	**0.107**

Duration of treatment

Duration of treatment shows no significant difference between responders and none responders (chi-squared test p=0.103) **(Table 3.2.5)**.

Table 3.2.5. Number of days by response to radiotherapy:

	Failed treatment	**Enduring complete response**	**p-value**
Duration =< 33 days	40	33	**0.103**
Duration > 33 days	19	9	

3.2.2. The effect of tumour stage, tumour grade and site in the bladder on the response to EBRT:

Tumour stage

48 (47.5%) of the tumour were stage 2 and 53 (52.4%) were stage 3. There was no significant difference in the response to EBRT between the two stages (chi-squared test p=0.827) **(Table 3.2.6).**

Table 3.2.6. Stage of tumour by response to EBRT:

	Failed treatment	Enduring complete response	p-value
Stage 2	27	21	**0.827**
Stage 3	32	21	

Tumour grade

11 tumours (10.9%) were grade 2 and 90 (89.1%) were grade 3. There was no significant difference between the two grades and their response to EBRT (chi-squared test p=0.486) **(Table 3.2.7).**

Table 3.2.7. Grade of tumour by response to EBRT:

	Failed treatment	Enduring complete response	p-value
Grade 2	8	3	**0.486**
Grade 3	51	39	

Site of the tumour

Tumours were defined as occurring on the right wall (25), the left wall (30) or "other" sites (46). No significant association was found between the site of the tumour and the response to radiotherapy (chi-squared test p=0.293) **(Table 3.2.8).**

Table 3.2.8. Site of tumour by response to EBRT:

	Failed treatment	**Enduring complete response**	**p-value**
Right wall	14	11	**0.293**
Left wall	21	9	
"other"	24	22	

3.2.3. Tumour angiogenesis, Intratumour macrophage infiltration,p53, MIB-1, bcl-2 and the response to EBRT

Angiogenesis

59 patients did not respond to radiotherapy. The median MVD for these patients using CD31 was 4.30 (IQR 2.00) and using CD34 was 4.60 (IQR 1.40). 42 patients did respond to radiotherapy and their median MVDs' were 4.30 (IQR 1.78) and 5.00 (IQR 2.30) respectively. No statistically significant association was found between MVD count and the response to EBRT for either method of quantifying angiogenesis using Mann-Whitney test [(p=0.635 (CD31) and p= 0.438 (CD34)] **(Table 3.2.9).**

Table 3.2.9. Median (IQR) of tumour angiogenesis by response to radiotherapy:

	EBRT	Median MVD (IQR)	p-value
MVD (CD31)	Failed treatment	4.30 (2.00)	**0.635**
	Enduring complete response	4.30 (1.78)	
MVD (CD34)	Failed treatment	4.60 (1.40)	**0.438**
	Enduring complete response	5.00 (2.30)	

Intratumour macrophages

Macrophage infiltration (CD68) was detected in all the specimens. The CD68 labelling index (LI) ranged from 23.8% to 67.2% (mean 41.43%, SD 10.20) for none responders and 20% to 65.4% (mean 43.81, SD 11.38) for patients who did respond to EBRT. The mean CD68 labelling index showed no significant difference between patients who did not respond to EBRT and those who did (t-test p= 0.273) **(Table 3.2.10).**

Table 3.2.10. Mean (SD) of CD 68 (LI) infiltrations by response to radiotherapy:

EBRT	Mean CD68 (SD)	p-value
Failed treatment	41.43 (10.20)	**0.273**
Enduring complete response	43.81 (11.38)	

p53 expression

Nuclear p53 protein was detected in 90 tumours. 11 of the tumours stained negative for p53. The p53 labelling index (p53 LI) ranged from 0% to 99% (median 63.50, IQR 36.00) for the no response group of patients and from 0% to 99% (median 61.45, IQR 47.70) for tumours in the response group. The median p53 LI showed no significant difference between tumours that responded to EBRT and those, which did not. Using a Mann-Whitney test (p=0.206) **(Table 3.2.11).**

Table 3.2.11. Median (IQR) of p53 (LI) infiltrations by response to radiotherapy:

EBRT	Median p53 LI (IQR)	p-value
Failed treatment	63.50 (36.00)	**0.206**
Enduring complete response	61.45 (47.70)	

MIB-1

The MIB-1 LI of tumours ranged from 32.7% to 98.6% (mean 68.25, SD 15.35) for tumours, which did not respond, to radiotherapy and from 33.8% to 96% (mean 65.82, SD 16.20) for tumours, which did respond. There was no significant difference in the MIB-1 LI between the two groups (t-test p=0.446) (Table 3.2.12).

Table 3.2.12. Mean (SD) of MIB-1 (LI) by response to radiotherapy:

EBRT	Mean MIB-1 LI (SD)	p-value
Failed treatment	68.25 (15.35)	**0.945**
Enduring complete response	65.82 (16.20)	

Bcl-2

48 (47.5%) of the tumours showed bcl-2 expression and 53 (52.5%) no expression of bcl-2. The response of the two groups to EBRT is shown in the following table. No significant association was found for bcl-2 scoring and the response to EBRT (chi-squared test p=0.320) **(Table 3.2.13).**

Table 3.2.13. Response to radiotherapy by bcl-2:

	No response	Enduring complete response	p-value
Bcl-2 +ve	31	17	**0.320**
Bcl-2 -ve	28	25	

3.2.4 Multivariate analysis of the effects of the variables with respect to the response to radiotherapy:

A multivariate logistic regression was fitted to the data with response to radiotherapy as the dependant variable and the other variables as independent.

The table below shows an output of the backward stepwise logistic regression. Sex and p53 are the only variables selected **(Table 3.2.14).**

Table 3.2.14. Predictors for response to EBRT:

	B	Sig.	Odds ratio	95.0% C.I. for OR	
				Lower	Upper
[a] Sex	-1.906	0.017	0.149	0.031	0.707
p53	-0.014	0.073	0.986	0.972	1.001
Constant	2.574	0.011	13.123		

[a] Variable(s) entered on step 1: Age, Grade, HB, Site, Stage, CD31, CD68, Days, Sex, Radioth. Dose, Bcl-2 and p53.

4. Survival

Survival analysis describes the analysis of data that correspond to the time from a well-defined time origin until the occurrence of some particular event or end-point. If the end-point is the death of a patient, the variable of interest will be survival times.

Because the cause of death is not recorded in these data, only the crude survival time of patients is considered. The 5-year survival in this study was 32%.

Kaplan-Meier survival plots (K-M plots) and log rank test were used to show difference between survivals for different factors.

The table (4.1) and figure (4.1) show a description of the variable, number of days of treatment. The mean value is 33.21 days with 95% confidence interval (31.83 to 34.59). 33 days can be used as a cut-off point as it falls within the confidence interval.

The Kaplan-Meier survival analysis shows that the survival time is significantly higher for those who were exposed longer to radiotherapy (> 33 days) (Log Rank, p=0.0246) (Fig. 4.2). The analysis showed significance for cut-off points between 30 and 40 days but not below or above these limits. This can be explained by the fact that few cases are observed on both tails of the distribution of days, which makes the size of one of the groups very small, and therefore reduce the power of the test (log rank), which is based on the chi-square test.

The same procedures for cut-off points have been used for the remaining variables (table 4.1).

CD68 shows a significantly higher survival time for those who have CD68 higher than 42.4 (log rank, p=0.036) (fig. 4.3).

The rest of the K-M plots (**Figure 4.4 - Figure. 4.12**) show no difference in survival times.

Table 4.1. Cut-off points of the variables:

			Cut-off point*
Duration of treatment (days)	Mean*		33.2
	95% Confidence interval for mean	Lower bound	31.8
		Upper bound	34.6
Tumour Angiogenesis CD31	Mean*		4.4
	95% Confidence interval for mean	Lower bound	4.2
		Upper bound	4.6
Tumour Angiogenesis CD34	Mean*		4.6
	95% Confidence interval for mean	Lower bound	4.3
		Upper bound	4.8
CD68	Mean*		42.4
	95% Confidence interval for mean	Lower bound	40.3
		Upper bound	44.5
Haemoglobin level	Mean*		12.8
	95% Confidence interval for mean	Lower bound	12.5
		Upper bound	13.1
MIB-1	Mean*		67.2
	95% Confidence interval for mean	Lower bound	64.1
		Upper bound	70.3
p53	Mean*		57.37
	95% Confidence interval for mean	Lower bound	51.75
		Upper bound	63.00

Figure 4.1. Mean (SD) for length of treatment:

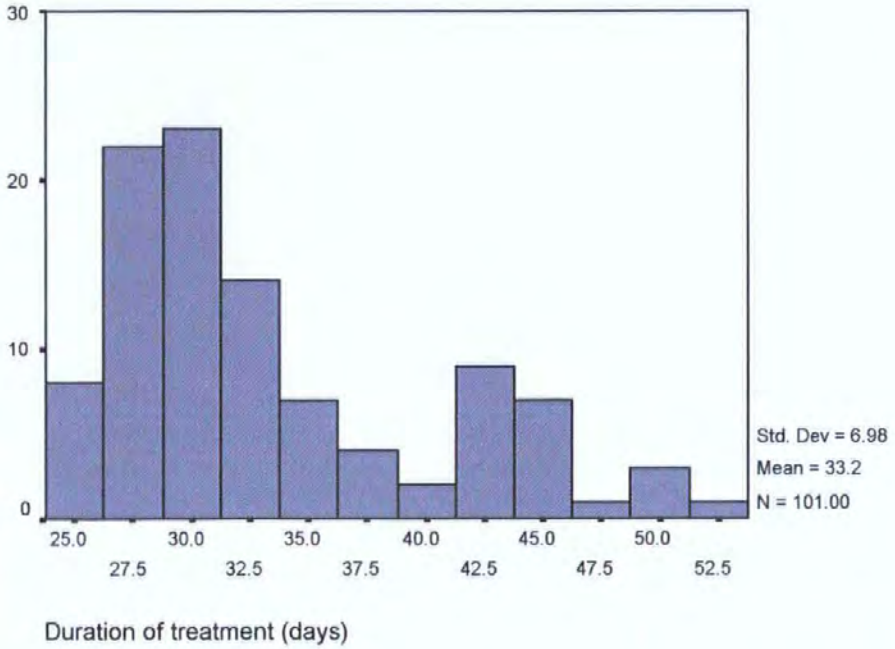


Figure 4.2. Survival by length of treatment:

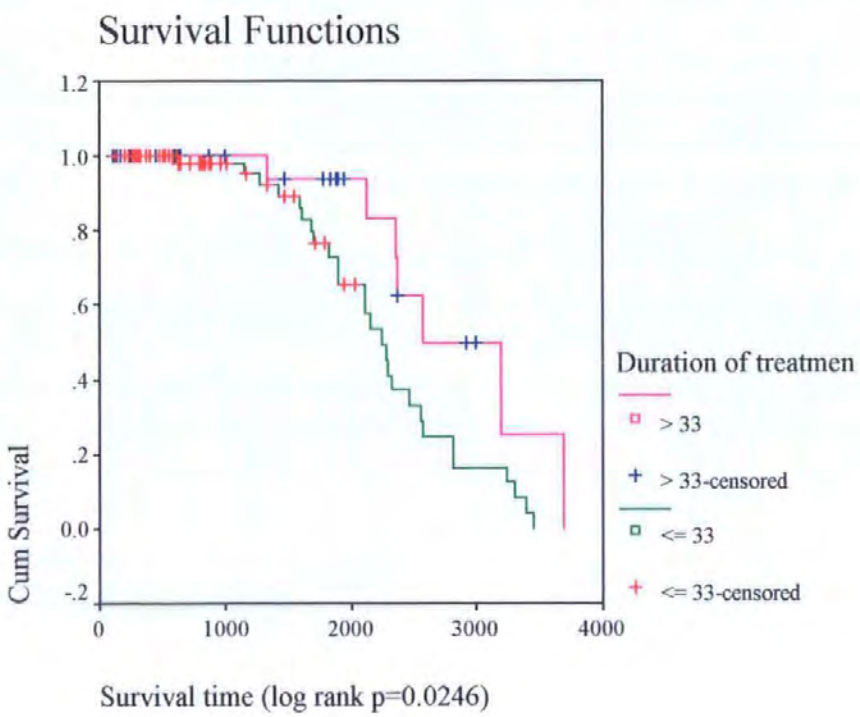


Figure 4.3. Survival times for CD68 (macrophage):

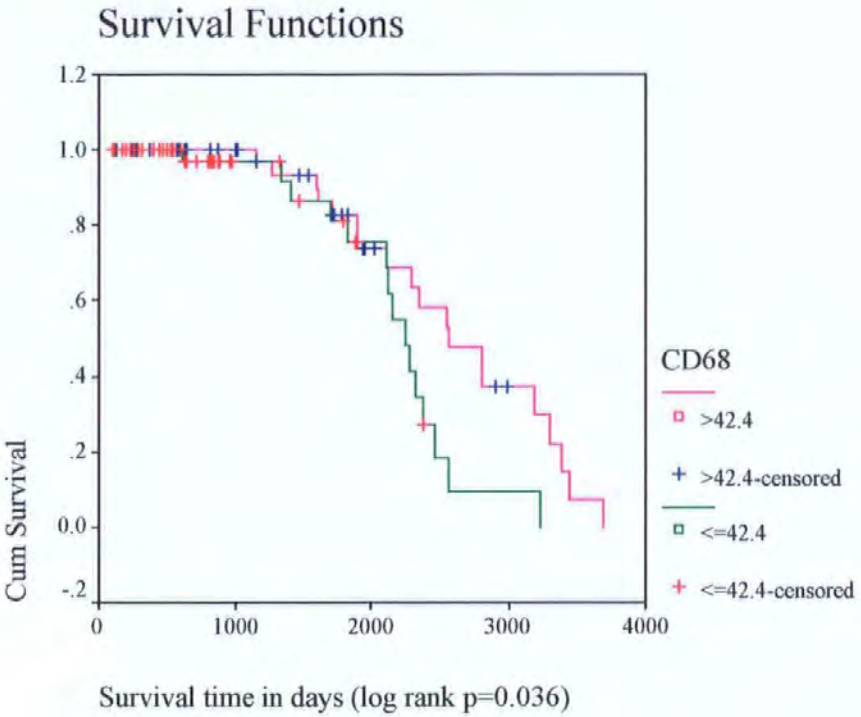


Figure 4.4. Survival times for stage of tumour:

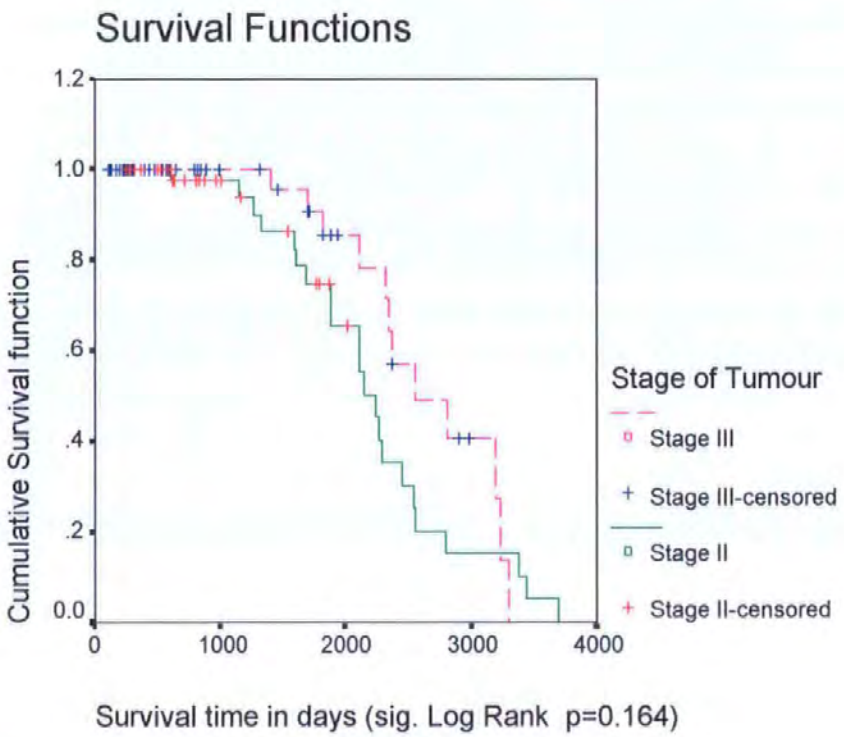


Figure 4.5. Survival times for grade of tumour:

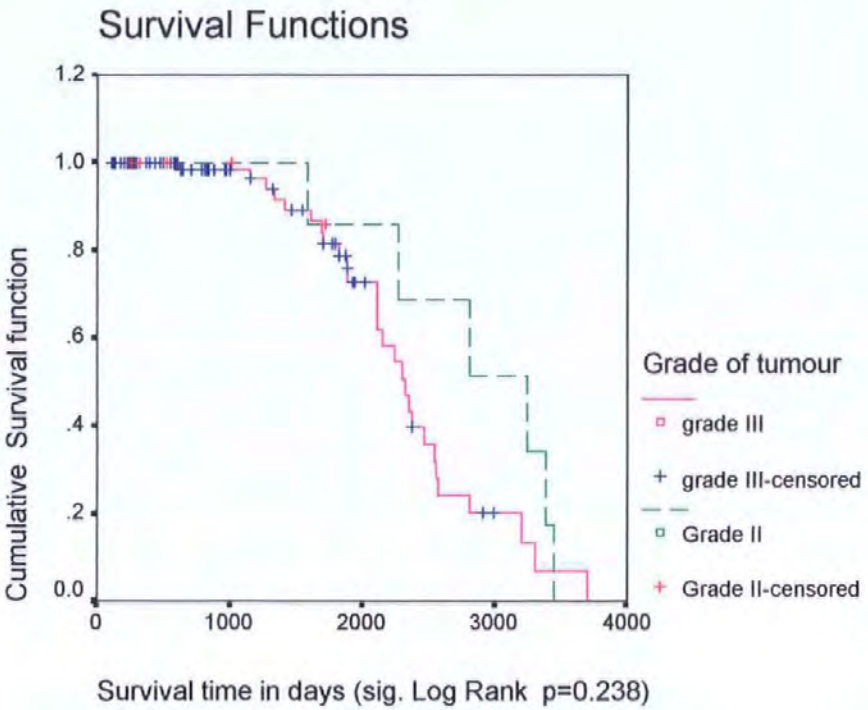


Figure 4.6. Survival times for Haemoglobin level:

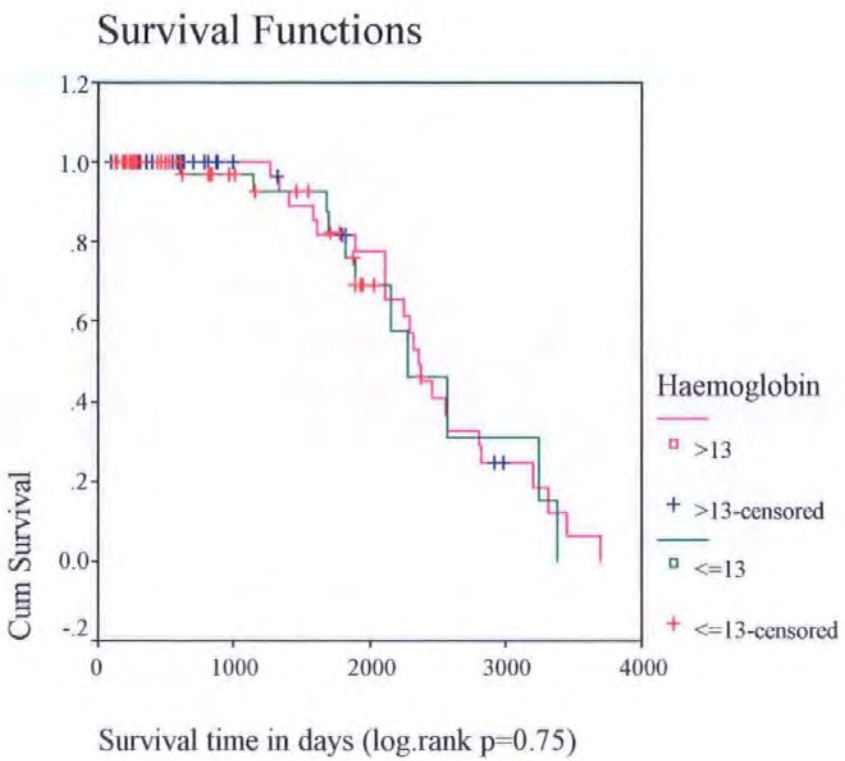


Figure 4.7. Survival times for CD31:

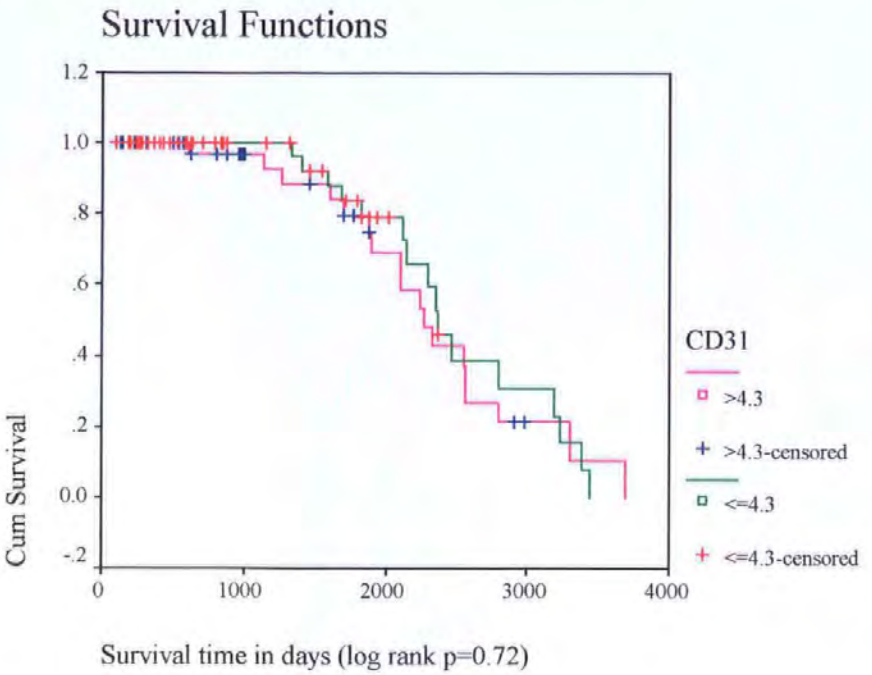


Figure 4.8. Survival times for CD34:

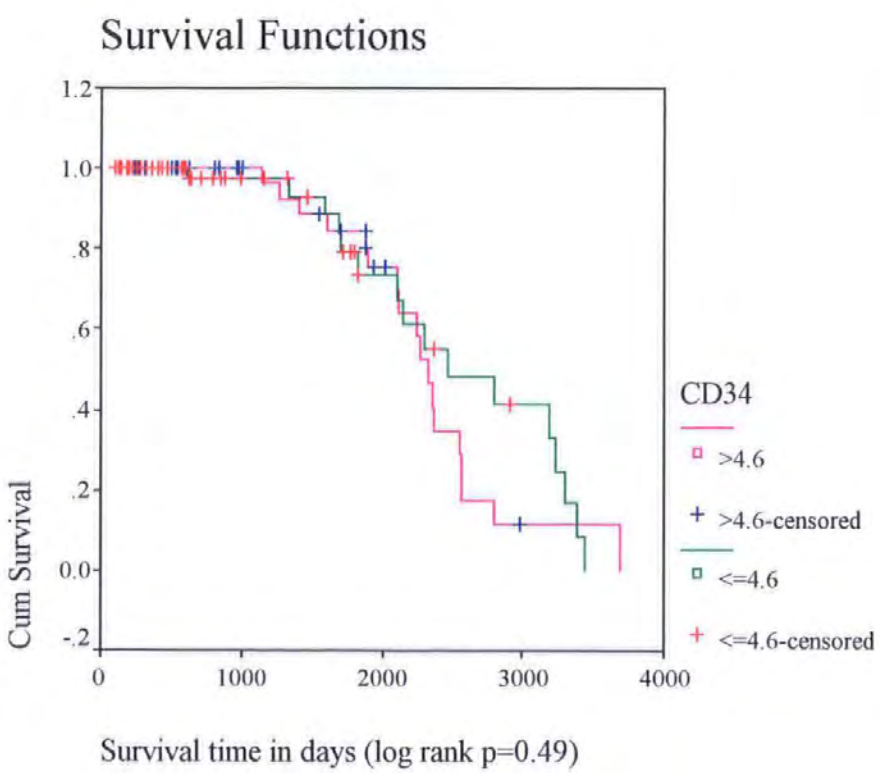


Figure 4.9. Survival times for gender:

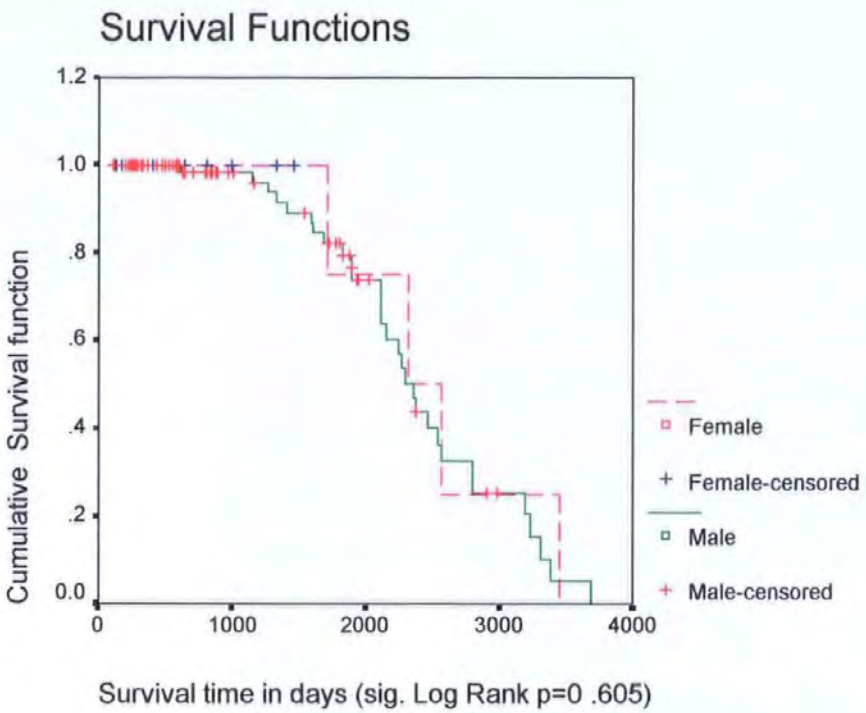


Figure 4.10. Survival time by tumour proliferation (MIB-1):

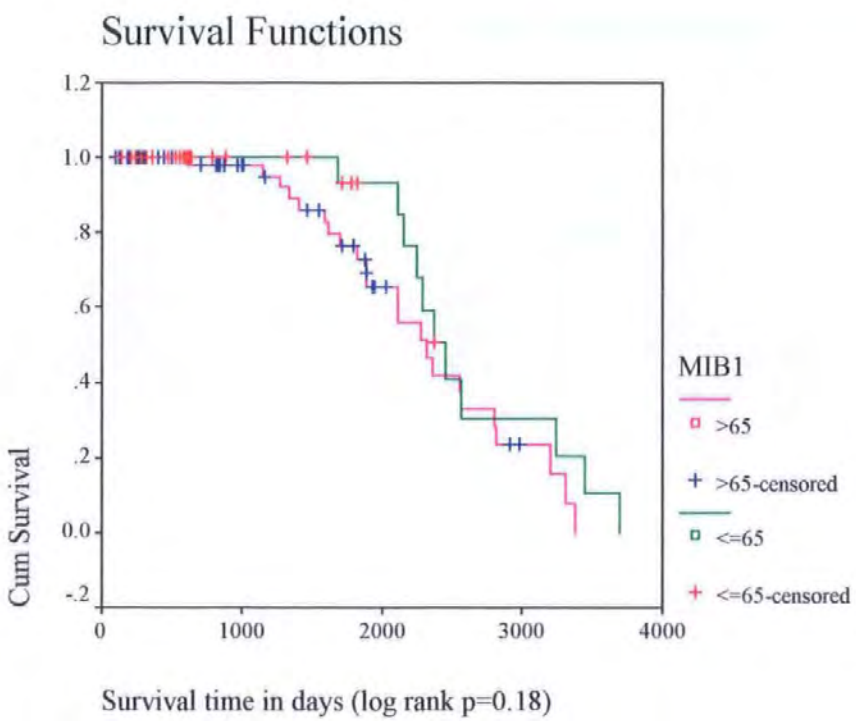
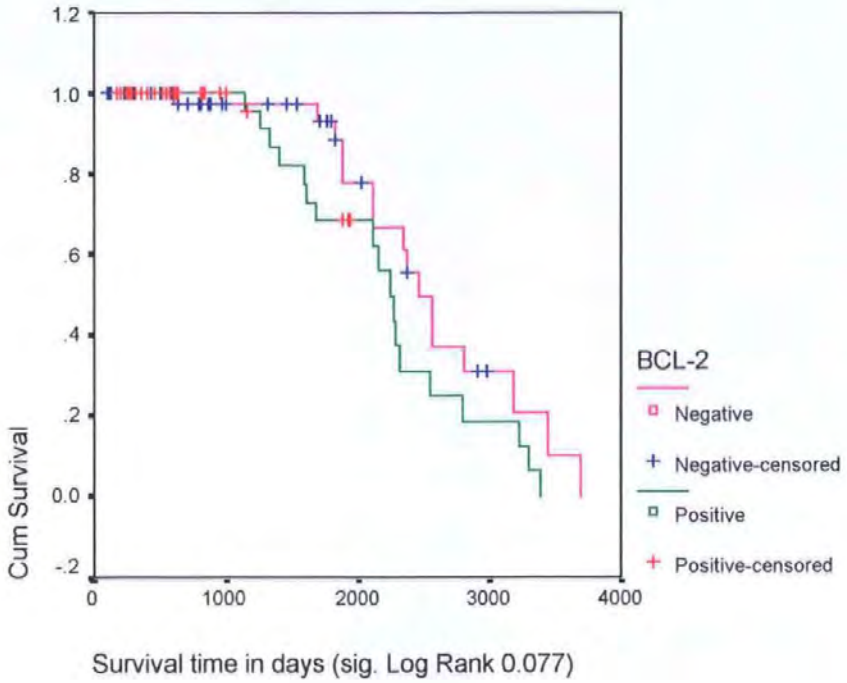
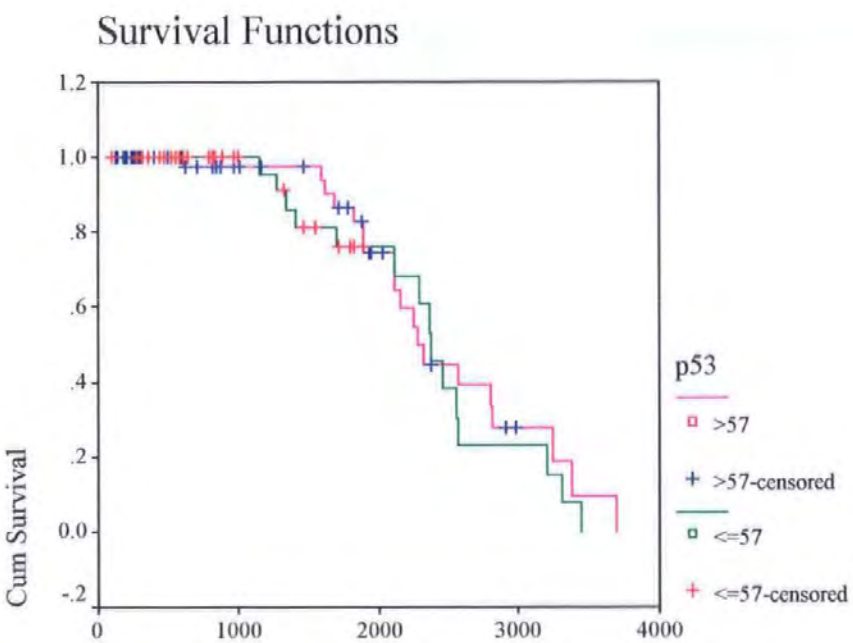


Figure 4.11. Survival time by bcl-2:



Survival time in days (sig. Log Rank 0.077)

Figure 4.12. Survival time by p53:



Survival time in days (log rank p=0.56)

CHAPTER FOUR

GENERAL DISCUSSION
&
CONCLUSION

DISCUSSION

4.1 General discussion

The expansion of knowledge in cancer biology has transformed our understanding of neoplasia. It is well recognized that solid tumour carcinogenesis is dependent upon both increased cellular proliferation as well as reduced cellular death. The recognition of this interplay of various factors has potentially both prognostic and therapeutic implications.

In this extensive study of 101 patients with muscle invasive transitional cell carcinoma treated by EBRT, several biological parameters associated with either cell proliferation or cell death were examined as potential prognostic indicators. Various physiological factors including age, sex, clinical staging, grading, site of tumour, haemoglobin level, radiotherapy total dose and duration of treatment, were also examined. Regulators of the apoptotic pathway, bcl-2 and p53 are recognized to be prognostic markers in several tumours and our study evaluated bcl-2 and p53 expression (as indicated by immunohistochemistry) as potential prognostic markers for evaluating bladder tumour response to radiotherapy. In all 101 cases pre-treatment bladder tumour tissue was analysed by a blinded, stringent histological scoring system for each antibody.

The most important aim of this study was to try and identify markers, which would predict the response of muscle invasive TCC of the bladder to EBRT. In this study, none of the molecular cell markers examined were found to be predictors of response in either univariate or multivariate analysis. Furthermore the response did not appear to be associated with age, and duration of treatment, stage and grade of the tumour or the site of the tumour in the muscle invasive bladder TCC. In all the statistical analysis females

were found to respond less well than males and haemoglobin level found to be higher for response group.

The data obtained have been carefully analysed and several interesting observations made.

p53

In 89% of tumours (90 out of 101), nuclear p53 was detected by IHC. Statistical analysis of the median p53 labelling index failed to show a significant difference between those tumours that responded to EBRT and those, which did not. Also there was no statistical correlation between the p53 nuclear labelling index and tumour grade and stage in this study.

The immunohistochemical determination of p53 status has a number of limitations. One of the difficulties in interpreting the results of p53 staining is the general lack of consistency in the technical aspects of performing the assay; another difficulty is in the interpretation of the results, since threshold values for altered p53 expression vary among studies. These technical considerations are the subject of a multicenter National Cancer Institute-sponsored trial evaluating the reproducibility of the interpretation of p53 staining between different laboratories that routinely performs the assay (McShane *et al.*, 2000). Although the overall interlaboratory reproducibility for the interpretation of p53 staining was good for specimens with no or minimal staining or for specimens with a high number of stained tumour cells, the reproducibility was not good for specimens with an intermediate range of staining. Such findings again indicate the need for caution when comparing results from different laboratories.

In theory, the inactivation of p53 may be seen in either chromosomal loss of 17p 13.1 and/or preferential mutations. The relationships between p53 protein over-expression as detected by immunostaining and p53 mutation have been seen in many solid tumours

including urothelial carcinoma. However, increased p53 immunoexpression may also be caused by p53 protein stabilization, for example by its binding to other proteins such as mdm2 (Haupt *et al.*, 1997). The mdm2 protein inhibits G1 arrest and the apoptotic functions of p53 protein by stabilizing and inactivating wild type p53 protein (Chen *et al.*, 1996).

In cases of nonsense splicing variants and stop-codon mutations (Bodner *et al.*, 1992) p53 may be mutated without being detected by IHC. As sequence analysis was not performed in this study, we cannot exclude the possibility that normal expression did not hide a mutation, not picked up by IHC. Conversely, p53 positive staining may have been caused by the over expression of abnormally abundant, but normally functioning protein (Kociakowski *et al.*, 1995). Also, the fixation of the archival material and the choice of antibodies may also affect the results (Fischer *et al.*, 1994).

We chose antibody DO7 as this is a widely used antibody, so that our study would be comparable to others in the literature.

However, ignoring potential technical limitations of p53 immunostaining this studies results raises several interesting points.

Kim *et al.*, in 1997 recently provided evidence in support of alternative pathways for radiation-induced the apoptosis. It has been well known that p53-independent apoptosis pathways exist in different cell types. For example pathways for p53-dependent (radiation-induced) and p53-independent (steroid induced) apoptosis have been identified in T-lymphocytes and thymocytes. Nevertheless, multiple pathways for radiation-induced apoptosis have not yet been characterised with most studies documenting the need for functional p53 in that pathway. However, recently, studies have shown that platelet-derived growth factor (PDGF) may mediate a p53-independent pathway by which radiation mediates apoptosis (Kim *et al.*, 1997). This finding may, therefore, explain why there was no correlation between tumour response to

radiotherapy and p53 immunostaining. Secondly, p53 mutation alone might not be enough to influence treatment outcome, as carcinogenesis is a multistep process and many more oncogenes are involved, each of them with a possible influence on treatment outcome. Wu *et al.*, 1996; Osen *et al.*, 1998; Qureshi *et al.*, 2001 also found no significant relationship observed between p53 detected by IHC and radiation response in patients with muscle invasive bladder cancer. However, Moonen *et al.*, 2001, in a more recent study established a significant relation between mutant p53 positivity and poor local control. In fact, conflicting results regarding the significance of many biological markers including p53 in relation to progress of the disease and response to treatment were established in many studies (Ong *et al.*, 2001; Rodel *et al.*, 2000; Krupski *et al.*, 2000; Pfister *et al.*, 1998; Popov *et al.*, 1997; Jahnson *et al.*, 1995).

p53 tumour suppressor gene mutation has been described as occurring frequently in invasive and in-situ bladder tumours. High grade and high stage tumours were often found to be positive for p53 (Nakopoulou *et al.*, 1998). However, others have failed to show this correlation (Cooke *et al.*, 2000, Wunderlich *et al.*, 2000).

In this study only 11 tumours (11%) were grade 2 whilst (90) 89% were grade 3. The failure to detect a significant difference between the p53 immunostaining for these 2 grades may have been caused by the small number of grade 2 tumours examined. 48 tumours (47%) of tumours were stage 2 and 53 (53%) were stage 3, and by definition, these are high grade aggressive tumours, therefore, a significant p53 variation would be unlikely.

Finally, the picture is complicated by the fact that immunohistochemical detection of oncoproteins not always reflects the functional status of the gene. For instance, it has been demonstrated that p53 stabilization is not necessarily a result of mutation-altered conformational changes but might as well be related to the tumour environment (Lane, 1994). Furthermore, missense mutations often cause p53 protein overexpression, but

immunohistochemistry is unable to detect other mutations that prevent protein expression. Greenblatt *et al.*, has pooled data from 84 studies that reported immunohistochemistry and DNA sequencing, and found that the sensitivity of detecting mutations by immunohistochemistry was only 75% and that the positive predicting value was only 63%. Future studies should be, therefore, preferentially performed with full knowledge of both protein and gene status (Greenblatt *et al.*, 1994).

Bcl-2

Apoptosis is a complex pathway involving the interaction of numerous biologically active proteins and peptides. The protein encoded by the bcl-2 proto-oncogene is one such entity and this has been implicated in prolonging cell survival by blocking apoptosis. Cytoplasmic bcl-2 is thought to antagonise radiation-induced apoptotic pathways by inhibiting the release of cytochrome C from the mitochondria (Hockenbery *et al.*, 1991). Increased expression of bcl-2 has been shown to alter tumour sensitivity to both chemotherapy and radiotherapy regimes (Lotem *et al.*, 1993). However, in contrast, Rodel *et al.*, 2000 found the expression of bcl-2 had no important influence on response to a combination of platin-based chemotherapy and radiation. A study carried out by Kong *et al.*, concluded that bcl-2 expression correlated with a poorer response to radiation treatment, which indicates that a poorer prognosis for patients with bcl-2 expression in invasive tumour could be partly due to the resistant response of cancer to radiation treatment (Kong *et al.*, 1998). This is consistent with the hypothesis that bcl-2 protein could contribute to the resistance of epithelial cancer to clinical treatment, which has been observed in prostate carcinoma. Bcl-2 expression in prostate cancer is associated with tumour progression after hormonal therapy, suggesting that bcl-2 may increase resistance to androgen ablation treatment (McDonnell *et al.*, 1992). Pollack *et al.*, also reported that bcl-2 overexpression was associated with impaired radiation

response in patients with bladder cancer treated with a regimen of preoperative radiotherapy (Pollack *et al.*, 1997).

However, whilst bcl-2 over-expression in certain types of malignancies such as high grade lymphomas, leukemia, neuroblastoma and prostatic cancer is a poor prognostic factor, lung and breast cancer patients with bcl-2 expression have a better chance of survival (Lu *et al.*, 1996). It has been argued that these differences could be due to multiple factors including tumour heterogeneity, tissue specificity, the evaluation method used in assessing bcl-2 expression, or most importantly the actual cause of the bcl-2 over expression In many tumours there is the classic t(14:18) translocation, but this has not been detected in bladder carcinoma, and suggestions of complex post transcriptional regulation may play a significant role in controlling the expression of bcl-2 (Yin *et al.*, 1994). Bcl-2 overexpression in bladder cancers is most commonly seen in early low grade and low stage tumours, whilst loss of bcl-2 expression is significantly correlated with high TCC stage, particularly in invasive TCC. This is thought to be due to the presence of overexpressed bcl-2 prolonging cell survival by inhibiting apoptosis and therefore leading to neoplastic growth at a slower rate.

In this study a surprising 48 tumours (48%) showed bcl-2 expression whilst 53 tumours (52%) did not. Eight out of 11 positive tumours (73%) were grade 2 TCC whilst 40 bcl-2 positive tumours out of 90 (44%) were grade 3 TCC. Statistically no significant difference between bcl-2 positivity and tumour grade has been found but there does seem to be a trend that fewer higher grade tumours, are bcl-2 positive as was found by Nakopoulous *et al.*, 1998.

Forty six out of 101 tumours (45.5%) did not respond to radiotherapy and this included 25 out of 48 tumours (54%) of bcl-2 positive tumours. Of the 55 out of 101 tumours that did respond this included 23 out of 48 tumours (48%) of bcl-2 positive tumours. Whilst we found no statistical significant evidence that bcl-2 expression is a useful

prognostic indicator to assess response to radiotherapy, the results do suggest a trend that bcl-2 positive tumours are less likely to respond to radiotherapy. There may be many reasons why so many high stage and high grade TCCs in this study are bcl-2 positive and have responded to radiotherapy either in the short or long term. There may be other factors affecting the response of tumour to radiotherapy, we have already discussed the role of p53 and there are numerous complex interactions required for the inhibition of apoptosis, not touched on by this study such as the subsequent heterodimerisation of bcl-2 with Bax, that requires the BH1 and BH2 domains of bcl-2.

Reported data on the relationship of bcl-2 expression with standard clinicopathological parameters in urothelial carcinomas have so far been conflicting. Most studies, including the present one, have failed to establish an association between the presence of bcl-2 expression and grade or stage (Korkolopoulou *et al.*, 2002; Wu *et al.*, 2000; Kirsch *et al.*, 1998; Tzai *et al.*, 1998). There are also studies documenting an inverse or a positive association of bcl-2 expression with grade and/or stage (Nakopoulou *et al.*, 1998; Okamura *et al.*, 1998; Li *et al.*, 1998; King *et al.*, 1996 & Bilim *et al.*, 1996).

The data showed no association between bcl-2 and p53 expression, which supported the finding of Cooke *et al.*, 2000 and Ong *et al.*, 2001. They found no relationship between expression of the two proteins on the tumours of patients with muscle invasive TCC.

No significant correlation between bcl-2 and MIB-1 was found in these data. Rodel *et al.*, 2000, reported similar results. Although, Rodel *et al.*, in his work patients were treated for invasive bladder cancer by a combined treatment protocol, including transurethral resection (TUR-B) and radiochemotherapy (RCT).

No correlation was found between bcl-2 and tumour angiogenesis and macrophages infiltration in this study.

MIB-1

Ki-67, a DNA binding protein is associated with cell proliferation and is expressed in all active phases of the cell cycle (Schwarting *et al.*, 1993) and Ki-67 labelled cells are recognised as a measure of the growth fraction. Characterisation of the protein recognised by Ki-67 antibody has led to the development of new monoclonal antibodies such as MIB-1, which advantageously can be used in formalin-fixed paraffin embedded tissue. Immunohistochemical determination of proliferative activity using MIB-1 equates with Ki-67 staining and has been demonstrated to be related to tumour biology, including tumour recurrence, tumour stage and grade in a given cell population.

This study evaluated the MIB-1 LI as a potential prognostic indicator for muscle-invasive TCC treated by EBRT. No statistical significance was found between the mean MIB-1 LI and those tumours that did and did not respond to radiotherapy. The range of values for a tumour that did not respond lies between 32.7%-98.6%, whilst the range for those that did respond is 33.8%-96%. This result is somewhat surprising as it would be expected that tumours with a high proliferative index (i.e. high growth fraction) would be more vulnerable to radiotherapy treatment. However, the index for the tumours that respond and those that do not is quite high and so other factors such as size of tumour, hence penetration of radiotherapy, blood supply etc. should also be considered. Another factor that must play a role in the interpretation of MIB-1 immunostaining is that proliferation in a neoplasm is a function of the proportion of cells replicating, as estimated by Ki-67 and of the time taken for completion of the cell cycle, a factor not measured by Ki-67. Therefore, a neoplasm with a slow cell cycle could have many cells in cycle, but still have a relatively low proliferation rate whereas a tumour with a short cell cycle could be highly proliferative with fewer cells actively cycling at any given moment (Kubota *et al.*, 1992).

The fact that MIB-1 immunostaining cannot measure the time spent in the cell cycle may explain the incomplete correlation between MIB-1 and the clinical course of muscle invasive bladder cancer.

A study conducted by Lara *et al.*, 1998 exhibited better local control after radical EBRT in bladder cancer patients with very low proliferating tumours indicating that rapid tumour repopulation during fractionated radiotherapy might reduce the probability of tumour control. However, Ogura *et al.*, 1995 showed that a high proliferative index predicted radioresponsiveness in 60 patients treated with pre-operative radiotherapy followed by radical cystectomy for invasive bladder cancer.

Moreover, Rodel *et al.*, 2000 also reported that the spontaneous AI and the proliferation index (Ki-67) helped to identify patients whose invasive bladder tumour responded completely to a regime of combined radiochemotherapy (RCT) following initial TURBT. In the data presented here a significant correlation between p53 LI expression and MIB-1 was observed. This is in agreement with the findings of Tsuji *et al.*, 1997 and Osen *et al.*, 1998. Conversely, Moch *et al.*, 1994 reported no significant differences between Ki-67 and p53 positive or negative tumour in muscle invasive bladder tumour.

No significance between MIB-1 immunostaining and grade and stage of tumour was observed in this study. Possibly, as discussed before, all the tumours in this study are of high grade and stage and therefore little significant information could be gleaned from examining these parameters. Other studies examining a wider range of TCC tumour grades and stages have reported discernible differences (Okamura *et al.*, 1990; Fontana *et al.*, 1992; Cohen *et al.*, 1993; Kruger *et al.*, 1995; Tsuji *et al.*, 1997 and Oosterhuis *et al.*, 2000).

Another factor that may influence the results and hence interpretation of the MIB-1 LI in this study, is the controversy regarding MIB-1 staining indices. Nakasu *et al.*, 2001

when examining meningiomas describes different results when using indices related either to randomly selected immunostained fields or to the area of highest labelling. There is no consensus on the better method to evaluate MIB-1 immunostaining though there is a commonly held believe that the degree of histological malignancy of tumours is determined by the area with the highest number of positive cells is commonly accepted.

CD31, CD34 and CD68

Tumour angiogenesis is essential for growth and metastases in human neoplasia. It is important for nutrient supply and production and delivery of various growth factors. Angiogenesis is usually determined by IHC staining of tumour tissue specific for endothelial cells. The degree of tumour angiogenesis is defined by the intratumoural microvessel density (IMD). This is known to be of prognostic value in many human malignancies, this circumstance being mainly attributable to the involvement of angiogenesis in the development of distant metastases. A considerable body of evidence points toward the existence of an interaction between tumour angiogenesis and radiation effect, either in terms of tumour oxygenation (Horsman and Overgaard, 1997), or of the vasculotoxic effects of X-rays (Baker and Krochak, 1989) or of the anti-apoptotic properties of angiogenic factors (Katoh *et al.*, 1995; Haimovitz-Friedman *et al.*, 1994; Fan *et al.*, 1998). Nonetheless, few clinical reports have assessed angiogenic activity as a predictive factor in radiation therapy and those that have are contradictory (Revesz *et al.*, 1989; Hall *et al.*, 1994; Gasparini *et al.*, 1995; Zatterstrom *et al.*, 1995; Giatromanolaki *et al.*, 1999). The predictive power of IMD for radiocurability may be explained in several ways. Hypoxia is a major cause of resistance to radiation (Horsman and Overgaard, 1997), and because oxygen delivery depends upon blood flow, measurement of tumoural vascularity has been deemed to represent a direct means of

assessing the tissue's oxygenation state (Revesz *et al.*, 1989; Awwad *et al.*, 1986). However, since hypoxia is a key trigger for the induction of angiogenesis (Dachs and Chaplin 1998), the number of microvessels could theoretically reflect the degree of tumour hypoxia. This view takes into account the circumstance that tumour vasculature is often dysfunctional, being characterized by sluggish blood flow or shunting (Jain, 1994). Moreover, high-resolution intravital measurements have revealed the absence of correlation between blood flow and perivascular pO_2 (Helmlinger *et al.*, 1997). Hence, the relationship between vascularity and the degree of oxygenation in clinical tumour samples remains to be clarified. Apart from the link between angiogenesis and hypoxia, angiogenic factors per se may interfere with radiosensitivity by inhibiting X-ray-induced apoptosis: VEGF is known to suppress γray-induced apoptosis in both CMK86 cells and normal haematopoietic stem cells (Katoh *et al.*, 1995). Furthermore, basic fibroblast growth factor has been shown to protect endothelial cells from radiation-induced apoptosis via a protein kinase C mediated mechanism (Haimovitz-Friedman *et al.*, 1994), and hepatocyte growth factor/scatter factor protects breast cancer cells from radiation-induced apoptosis by targeting the anti-apoptotic mitochondrial membrane protein Bcl-X_L (Fan *et al.*, 1998). Angiogenesis is closely regulated by a broad range of stimulators and inhibitors (Pepper *et al.*, 1996). p53, which is essentially involved in cell cycle control, DNA repair and the regulation of apoptosis (Agarwal *et al.*, 1998), also contributes to the regulation of angiogenesis: Loss of functional p53 leads to reduced expression of thrombospondin-1 (TSP-1) (Dameron *et al.*, 1994), a key inhibitor of angiogenesis (Tuszynski and Nicosia 1996). Mutant p53 has been shown to upregulate the expression of vascular endothelial growth factor (VEGF) (Kieser *et al.*, 1994), which has a pivotal role in triggering angiogenesis (Ferrara and Keyt, 1997). Two antibodies to endothelial cells were chosen. CD31 is recognized to be the most sensitive and specific endothelial cell marker (Miettinen *et al.*, 1994). With the notable

exception of macrophages and platelets, CD31 expression is not seen in any other non-endothelial tissue. Care was taken to distinguish between true endothelial CD31 immunostaining and that of macrophages. This is because the staining of macrophages is distinctly granular whilst the staining of endothelial cells is intensely cytoplasmic with linear membranous staining. The function of CD34, a transmembrane glycoprotein is unknown, however, it is recognised to stain non-endothelial cells including macrophages, haemopoietic stem cells and dendritic cells seen around blood vessels, nerve sheath and within the dermis (van de Rijn and Rouse, 1994).

This study showed that, the degree of angiogenesis and macrophages infiltration exhibited no marked difference in the average count of CD31, CD34, and CD68 in patients responded and those who did not respond to radiotherapy.

Both markers for angiogenesis (CD31&CD34) were expressed in all slides in this study. The median for CD31 count was 4.3 for responded patients and 4.3 for non-responded patients. Whilst the median for CD34 counts was 4.6 for responded patients and 4.6 for non-responded patients.

This study showed a strong positive Spearman correlation between MVD quantified by both CD31 and CD34, suggesting that both are reliable methods of quantifying angiogenesis in muscle invasive bladder TCC.

Staining with CD31 showed no association between MVD and stage, grade or site of the tumour. In the case of CD34 a statistically difference was found in MVD for tumour stage but not grade. Sagol *et al.*, 2001 reported increased MVD association with high grade of tumour. On the contrary, Krupski *et al.*, 2000 demonstrated that MVD did not significantly correlate with clinical stage while tumour grade did.

The data showed no association between MVD and p53 expression, which contradicts the finding of Bochner *et al.*, 1997. They found a significant association between p53 status and MVD in 161 cystectomy specimens.

No significant correlation between MVD and macrophage infiltration was found in these data. This is in contrast to the finding of Hanada *et al.*, 2000. These authors reported a positive correlation between TAM count and MVD. However, out of their total of 63 bladder cancer only 23 were muscle invasive tumours whilst the data presented here is from 101 muscle invasive tumours.

Increased MVD counts have been associated with tumour progression and decreased overall survival in many studies (Srivastava *et al.*, 1988; Barnhill *et al.*, 1992; Weidner *et al.*, 1992; Horak *et al.*, 1992; Weidner *et al.*, 1993; Brawer *et al.*, 1994; Bochner *et al.*, 1995; Jaeger *et al.*, 1995; Dickinson *et al.*, 1994; O'Brien *et al.*, 1995; Campbell *et al.*, 1997).

The data presented here do not support these finding as MVD did not predict either response to radiotherapy or overall survival. However, in this series it was only possible to analyse crude survival and not disease specific survival owing to incomplete clinical records.

There are important methodological considerations related to staining technique, which must be taken into account when using MVD in this way. Firstly, the method of antigen retrieval can markedly affect the visualisation of blood vessels in tissue sections. Secondly, the choice of primary anti-endothelial antibody (e.g. CD31, CD34 or vWF) can profoundly influence intensity of staining and thus blood vessel counts in tissue sections. In the past majority of investigators have used anti-vWF antibody, a pan endothelial marker, to quantify tumour MVD. It is now apparent that although this is excellent in visualising blood vessels in normal tissues, it may not stain some microvessels within and in the immediate vicinity of a tumour mass, which can be demonstrated by other endothelial markers (Wang *et al.*, 1994). Endothelial cells are highly heterogenous among normal vascular beds in their structure, function, growth

rate, antigenicity and pharmacology (Thorin *et al.*, 1997; Clarke *et al.*, 1997). These differences are even more pronounced between normal and tumour associated endothelial cells. This may be why anti-CD31, and not anti-CD34 or anti-vWF, proved a better reagent for staining blood vessels in bladder tumours. This is in contrast with observation made in colorectal tumours where it has been found that anti-CD34 antibody produced stronger and more localised staining compared with anti-CD31 antibody (Abdalla *et al.*, unpublished data). In other studies results of a panel of 5 anti-endothelial antibodies (CD31, CD34, CD105, vWF and PALE) have been compared to quantify MVD in breast carcinomas (Wang *et al.*, 1994). Anti-CD105 antibody was by far the best antibody in the visualisation of microvessels. If similar observation is made in other tumour types, it will have important practical implications, in that a single antibody may not be suitable reagent to visualise blood vessels in all types of tumours. For future studies we suggest that, prior to examining MVD in tumour, a panel of anti-endothelial antibodies should be screened to identify the one, which will reliably stain blood vessel in and around the tumour.

Angiogenesis was initially thought to be induced only by tumour cells themselves. However, it is likely that certain stromal components are also involved in the regulation of tumour behaviour. Among these, macrophages have been previously shown to play an important role in tumour angiogenesis as well as inflammatory reactions (Sunderkotter *et al.*, 1994; Leek *et al.*, 1994). It has been shown that TAM secrete a wide range of cytokines, many of which have direct growth promoting properties on tumour cells (Leek *et al.*, 1994; O'Sullivan *et al.*, 1993). Tumour associated macrophages also produce several angiogenic factors, such as VEGF and bFGF, suggesting that these cells may be involved in tumour vascularization and/or invasiveness via these cytokines (Lewis *et al.*, 1995; Scannel *et al.*, 1993).

CD68 expression was detected in all our slides and the CD68 labelling index (LI) ranged from 23.8% to 67.2% (mean 41.29%, SD 10.38) for none responders and 20% to 65.4% (mean 43.38, SD 11.00) for patients who did respond to EBRT. CD68 labelling index showed no statistically significant differences between patients responded and those who did not respond to radiotherapy.

The TAM count (CD68) did not predict stage or grade of the tumour or the response to EBRT in this study. We also found no positive correlation between TAM count and microvessel density count (CD31&CD34). These results do not support finding by Hanada *et al.*, 2000 and Leek *et al.*, 1996. They observed a positive correlation between TAM count and microvessel density count.

The TAM count did however significantly predict crude survival, patients with higher counts (> 42) having a longer survival (Kaplain-Meir, p=0.036). Our results suggest that determination of TAM count in muscle invasive bladder cancer tissues is of value to predict the survival in patients with bladder cancer. This is the opposite of the findings of Hanada *et al.*, 2000. These authors found that the 5 year survival rate was significantly worse in patients with higher TAM counts.

Treatment duration

Reducing overall treatment time is another approach founded in the radiobiological results of tumour repopulation during the course of treatment (Hopkins and Looney 1987). Split course in other tumour sites has been abandoned because longer treatment time has resulted in decreased tumour control (Overgaard *et al.*, 1988). This was not found in a randomised trial by Marcial *et al.*, 1985 and Salminen, 1989. They found no difference in response, long-term local control or survival. Decreased tumour control has been found in one retrospective study, where the 5 year survival of patients treated continuously were 35% in comparison to 22% when a split of 2-3 weeks were allowed

(Davidson *et al.*, 1990). This was approved in another study in which data suggested that increased overall treatment time allowing accelerated tumour repopulation may decrease tumour control in transitional bladder cancer (Maciejewski *et al.*, 1991; Plataniotis *et al.*, 1994).

The data presented in this study show that survival time was significantly better in patients whose treatment was fractionated over a longer period (> 33 days).

Haemoglobin

Radiobiologic studies suggest that anemia may cause or contribute to resistance to radiation therapy by increasing the hypoxic tumour fraction (Gray *et al.*, 1953; Rubin and Casarett, 1968; Hirst, 1986). However, dose escalation to overcome the relative radioresistance of hypoxic cells is limited by the resultant risk of increased toxicity to the adjacent normal tissues. Measures aimed at overcoming tumour hypoxia that have been investigated include the use of hypoxic cell sensitizers (Dische, 1985; Overgaard *et al.*, 1989; Overgaard and Horsman, 1996; Grigsby *et al.*, 1999), hyperbaric oxygen (Dische and Anderson 1983), perfluorochemicals, and carbogen breathing (Song *et al.*, 1985), hypoxic cell toxins (Brown, 1993) or blood transfusions (Vigario *et al.*, 1973; Bush, 1984; Girinsky *et al.*, 1989; Fyles *et al.*, 1995; Pedersen *et al.*, 1995; Grogan *et al.*, 1999) and more recently, by treating with recombinant human erythropoietin (Vijayakumar *et al.*, 1993; Dusenbery *et al.*, 1994). Although blood transfusions have proven effective in raising haemoglobin levels and relieving clinical symptoms of anemia, their effect on prognosis is still unclear and controversial.

In some studies, the level of serum haemoglobin before therapy is considered as an indicator that should reflect the patient's general condition better than the performance status itself (Dunst *et al.*, 1994; Pollack *et al.*, 1994; Gospodarowicz *et al.*, 1989).

Pollack *et al.*, 1994 managed to prove the independent prognostic value of haemoglobin level for the overall survival as well as for the local control of disease in patients treated by cystectomy after previous radiotherapy, while the prognostic value of haemoglobin level in patients treated by radiotherapy alone was not clearly confirmed, considering the controversy of those reports (Pollack *et al.*, 1994; Gospodarowicz *et al.*, 1989). A group of investigators from Erlangen (Dunst *et al.*, 1994) has confirmed the prognostic value of haemoglobin in patients treated by conservative approach. In a study carried out by Matos *et al.*, 2000 that serum haemoglobin level failed to be associated with statistically significant differences in the disease specific survival.

In our study, serum haemoglobin level found to be higher in patients responded to EBRT. On the other hand, serum haemoglobin level had no significant effect on survival in our patients.

Gender

A surprise finding was that female responded less well to EBRT than male. Although appearing to be statistically significant, out of 101 patients in total only 11 were female and this finding should be interpreted with caution. Comparable results from Sumiyoshi *et al.*, 1994 that found the frequency of complete response was significantly different according to the sex (males 64%, females 29%; p=0.0239). Even though appearing to be statistically significant, patients were treated with a combination of intra-arterial chemotherapy and low dose radiotherapy.

There was an insignificant trend to longer overall survival in males, compared to females in this study. It would be interesting to look at a large numbers to see if this finding is of significance. Similar findings found in a study carried out by Scrimger *et al.*, 2001 and Harada *et al.*, 2000.

4.2 Conclusion

Although, muscle invasive transitional cell carcinoma of the bladder was intensively investigated in this study, none of the selected markers was proven to be a prognostic value in determining patients most suitable for radiotherapy as primary treatment with curative intent for their bladder cancer. Moreover, standardization and reproducibility are still major difficulties that hinder the clinical application of these biomarkers. However, their potential applicability cannot be ignored and should be investigated further. Whether these biological markers can be used as predictors of radiosensitivity in muscle invasive bladder TCC remain to be proven in larger and preferentially prospective studies.

The finding of a poorer response in females is also worthy of detailed study since, hormonal and anatomical influences may be important.

APPENDIX

RESULTS

Data was tested for normality using Kolmogorv-Smirnov test. Data with p value of >0.05 are normally distributed. The table below shows that only CD68 and MIB1 are normally distributed.

Test of Normality

	Kolmogorov-Smirnov
	p-value
CD31	0.001
CD34	0.007
CD68 %	0.200
p53 expression %	0.020
MIB-1 %	0.166
Haemoglobin level	0.031

Section one

The chi-square tests show no evidence of association between p53 positive or negative and response to EBRT (Tab. 1a&b).

Tab. 1a. p53 and response to radiotherapy

			Response to Radiotherapy		Total
			No response	Complete response	
p53	Negative	Count	2	9	11
			18.2%	81.8%	100.0%
	Positive	Count	44	46	90
			48.9%	51.1%	100.0%
Total		Count	46	55	101
			45.5%	54.5%	100.0%

Tab. 1b. Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	3.727	1	0.054		
Continuity Correction	2.591	1	**0.107**		
Likelihood Ratio	4.060	1	0.044		
Fisher's Exact Test				.062	.051

p53 and stage of tumour

There was no significant association between p53 (positive-negative) and tumour stage by means of chi-square (p=0.862) (Tab. 2a&b).

Tab. 2a. p53 by stage of tumour

		Stage of Tumour		Total
		Stage II	Stage III	
p53	Negative	6	5	11
	Positive	42	48	90
Total		48	53	101

Tab. 2b. Chi-Square Tests

	Value	Df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.244	1	.621		
Continuity Correction	.030	1	**0.862**		
Likelihood Ratio	.244	1	.622		
Fisher's Exact Test				.753	.430

p53 and Grade of tumour

There was no significant association between p53 (positive-negative) and tumour grade by means of chi-square (p=0.757) (Tab.3a&b).

Tab. 3a. p53 by Grade of tumour

		Grade of tumour		Total
		Grade II	Grade III	
p53	Negative	2	9	11
	Positive	9	81	90
Total		11	90	101

Tab. 3b. Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.676	1	.411		
Continuity Correction	0.096	1	**0.757**		
Likelihood Ratio	.589	1	.443		
Fisher's Exact Test				.342	.342

p53 (positive-negative) and angiogenesis

p53 (positive-negative) is not correlated (Spearman correlation) with CD31 (r=0.136, p=0.174) and CD34 (r=0.087, p=0.387) (Tab. 4a).

Tab. 4. Correlation between p53 (positive-negative) and MVD in bladder TCC:

		Tumour angiogenesis CD31	Tumour angiogenesis CD34
p53 (positive-negative) expression in bladder TCC	Correlation coefficient	0.136	0.087
	Sig. (2-tailed)	0.174	0.387
	N	101	101

p53 (positive-negative) and MIB-1

There was no significant Spearman correlation between p53 (positive-negative) and MIB-1 LI (r= -0.029, p=0.774) **(Tab. 5)**.

Tab. 5. Correlation between p53 (positive-negative) and MIB-1 in bladder TCC:

		Proliferation of tumour cells MIB1 %
p53 (positive-negative) expression in bladder TCC	Correlation coefficient	-0.029
	Sig. (2-tailed)	0.774
	N	101

p53 (positive-negative) and haemoglobin

p53 is not correlated (Spearman correlation) with haemoglobin level (r= -0.070, p=0.488) (Tab. 6).

Tab. 6. Correlation between p53 (positive-negative) and haemoglobin level in bladder TCC:

		haemoglobin level
p53 (positive-negative) expression in bladder TCC	Correlation coefficient	-0.070
	Sig. (2-tailed)	0.488
	N	101

p53 (positive-negative) and CD68

There was no significant positive Spearman correlation between p53 and CD68 (r= -0.006, p=0.951) (Tab. 7).

Tab. 7. Correlation between p53 and CD68 (LI) in bladder TCC:

		Tumour macrophage CD68
p53 (positive-negative) expression in bladder TCC	Correlation coefficient	-0.006
	Sig. (2-tailed)	0.951
	N	101

p53 and Bcl-2

No significant association was found between p53 (positive-negative) and Bcl-2 using Chi-Square tests (p= 0.862) (Tab. 8a&b).

Tab. 8a. p53(positive-negative) by Bcl-2.

		p53		Total
		Negative	Positive	
Bcl-2	Positive	6	42	48
	Negative	5	48	53
Total		11	90	101

Tab. 8b. Chi-Square Tests.

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.244	1	.621		
Continuity Correction	**.030**	**1**	**0.862**		
Likelihood Ratio	.244	1	.622		
Fisher's Exact Test				.753	.430

Section two

The chi-square test for association between EBRT and p53 (positive-negative) shows no statistical evidence of that association (Tab. 9a&b).

Tab. 9a. p53 by response to EBRT.

		Response to radiotherapy		Total
		Failed treatment	Enduring complete response	
p53	Negative	5	10	15
	Positive	54	32	86
Total		59	42	101

Tab. 9b. Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	4.562	1	.033		
Continuity Correction	**3.430**	**1**	**0.064**		
Likelihood Ratio	4.515	1	.034		
Fisher's Exact Test				.046	.033

p53 (positive-negative) has also been tested against stage and grade of tumour by means of chi-square but there were no significance (Tab. 10a&b, Tab. 11a&b).

Tab. 10a. p53 by grade of tumour

		Grade of tumour		Total
		grade II	grade III	
p53	Negative	2	13	15
	Positive	9	77	86
Total		11	90	101

Tab. 10b. Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.108	1	.742		
Continuity Correction	**.000**	**1**	**1.000**		
Likelihood Ratio	.103	1	.748		
Fisher's Exact Test				.666	.512

Tab. 11a. p53 by stage of tumour

		Stage of Tumour		Total
		Stage II	Stage III	
p53	Negative	7	8	15
	Positive	41	45	86
Total		48	53	101

Tab. 11b. Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.005	1	.943		
Continuity Correction	**.000**	**1**	**1.000**		
Likelihood Ratio	.005	1	.942		
Fisher's Exact Test				1.000	.583

Fig. 1. Survival time by p53: p53 has no effect on survival.

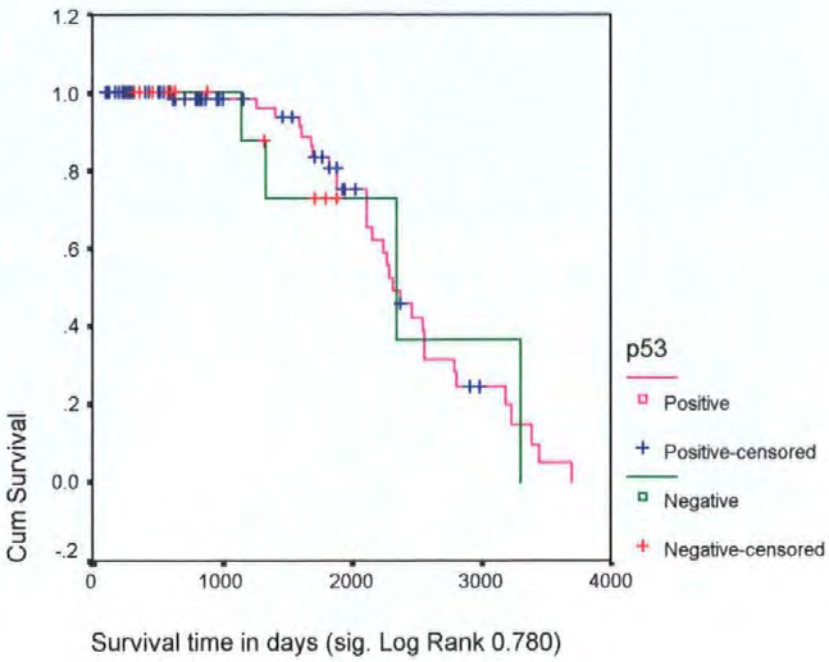


Fig. 2. Scatter plot of MIB-1 Vs MVD (CD34):

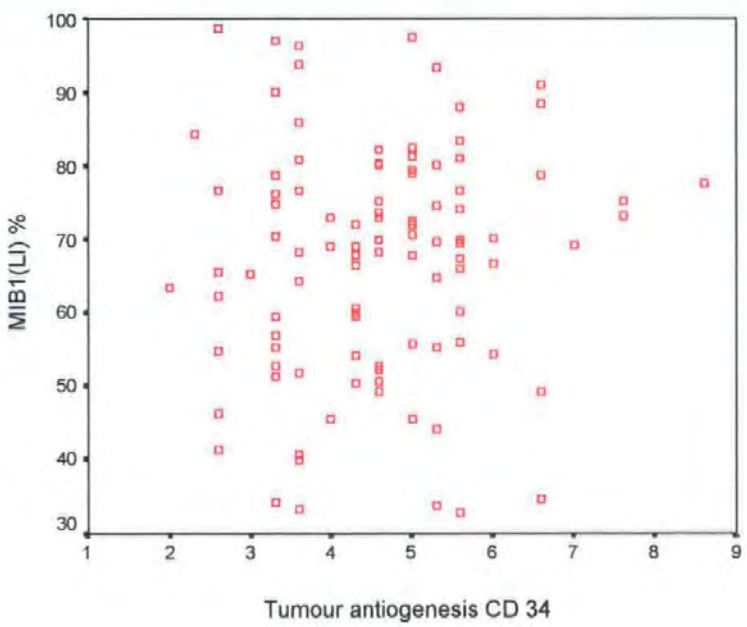


Fig. 3. Scatter plot of haemoglobin level Vs MVD (CD31):

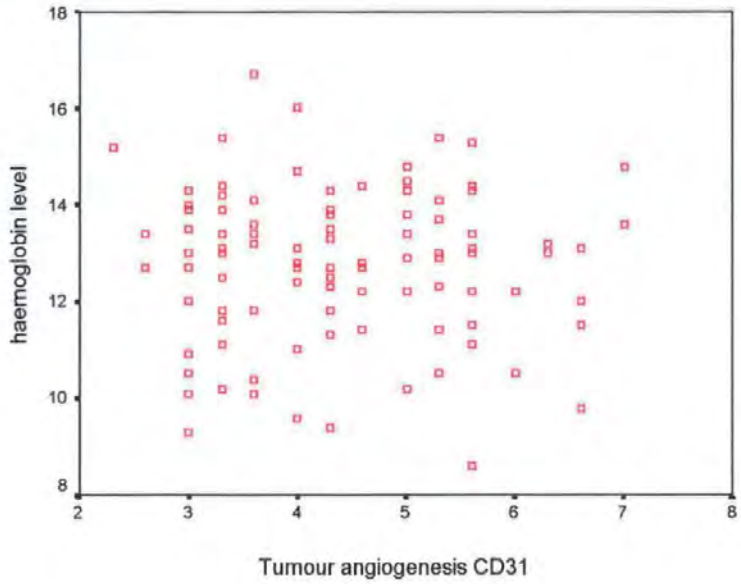
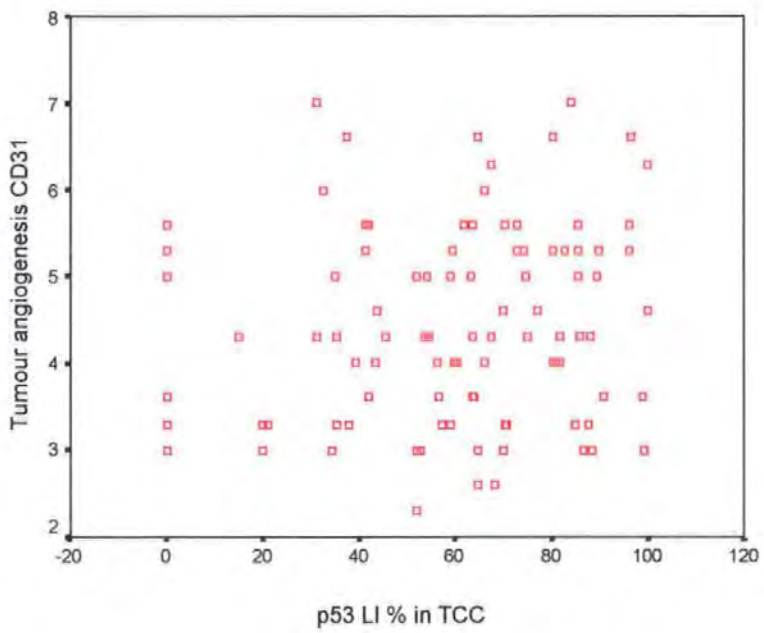


Fig. 4. Scatter plot of p53Vs MVD (CD31):



Numb.	Age	Sex	Stage	Grade	Radio.total dos.	Num. Frac.	Days	Cysto .Res.	Last point review	Start.Rad.	Alive	CD 31	CD 34	CD 68 %	bcl-2	p53 %	MIB1 %	Hb	Site
1	62	1	2	2	60	30	49	1	28.12.94	25.4.94	1	4	3.6	49.9	1	60.4	93.8	16	3
2	74	1	2	3	60	30	44	1	23.5.98	5.8.92	2	2.3	5	33.8	2	51.9	81.2	15.2	3
3	71	1	3	3	54	20	32	1	3.7.95	15.12.94	1	3.3	5.3	30.2	1	59.1	44.2	12.5	2
4	79	2	2	3	50	20	27	1	29.11.95	18.1.95	1	4.6	3.6	46.9	1	77.1	96.3	12.8	3
5	53	1	3	3	60	30	42	1	18.12.94	24.5.93	1	4.6	4.3	47	1	43.9	69	11.4	1
6	75	1	3	3	54	20	32	1	10.12.94	7.3.94	1	3	3.3	37.7	1	88.3	51.4	10.1	1
7	55	2	3	3	54	20	30	1	4.4.1995	1.3.94	1	2.6	3.6	40	1	64.5	68.2	13.4	2
8	73	2	3	3	50	25	38	1	22.3.94	28.9.93	1	4	3.3	30.4	1	81.6	96.9	11	2
9	76	1	3	3	50	25	32	1	28.2.94	16.11.92	1	3.3	3.6	32.1	1	21	64.2	11.1	3
10	76	1	3	3	54	20	27	1	30.9.92	3.6.92	1	5.3	4.6	34.9	2	95.9	72.8	12.9	3
11	75	2	3	3	54	20	27	1	30.9.94	8.7.92	1	6.6	6.6	47.7	1	96.2	91	9.8	2
12	50	2	3	3	54	20	32	1	17.9.98	7.5.92	2	5.6	5	25	1	85.4	67.8	14.4	1
13	78	1	3	2	60	30	43	1	15.8.93	9.3.92	1	5.6	5.3	30.2	1	41.5	33.8	11.1	1
14	76	1	2	3	54	20	25	1	20.11.98	24.2.92	2	3.3	3.3	34.9	2	37.7	34.1	15.4	3
15	81	1	2	3	60	30	41	1	1.3.93	13.6.91	1	5.6	6	67.2	1	63.5	54.3	13	2
16	70	1	2	3	54	20	28	1	25.1.94	10.6.91	1	5	5.3	35.6	1	85.5	74.5	12.9	2
17	70	1	3	3	60	30	49	1	28.9.91	19.2.90	1	5	3.6	57.9	2	59	40.6	13.8	2
18	56	2	3	3	58	29	42	1	19.11.93	16.11.89	1	3	3.3	60.8	2	51.9	76.2	13	3
19	74	2	3	3	60	30	44	1	6.10.89	21.3.89	1	4	4.6	36.7	2	59.7	69.9	9.6	3
20	64	1	3	3	60	30	44	1	19.10.97	16.8.89	1	5	5	48.6	2	63.3	79.3	13.8	3
21	74	1	2	3	54	20	29	1	10.2.92	19.9.89	1	6.3	4.6	50.2	2	99.8	82.1	13.2	2
22	55	2	3	3	58.8	29	31	1	19.11.93	16.11.89	1	5.3	4.3	41.8	2	82.5	59.4	13	1
23	64	1	3	3	60	30	45	1	24.11.88	15.8.88	1	3.6	4.3	32.1	2	56.4	71.9	13.2	3
24	74	1	3	2	60	30	45	1	24.1.90	10.3.89	1	3.3	2.6	26.5	2	0	46.2	14.4	1

25	46	1	2	3	60	30	43	1	18.9.91	27.4.89	1	3.6	4.6	39.8	2	98.8	80.4	13.4	2
26	73	1	2	3	52	20	28	1	12.4.1992	3.1.1990	1	3	5	41	1	52.8	70.6	10.9	3
27	69	1	2	2	52.5	20	25	1	3.5.99	24.1.1990	2	4	3.3	50	1	80.8	90	12.7	2
28	70	1	3	3	50	20	38	1	15.11.1990	28.3.1990	1	4.3	5.6	26	2	67.5	60.1	11.3	2
29	65	1	3	2	55	20	27	1	14.4.99	5.6.1990	2	3	4.3	37.6	1	70	50.4	9.3	3
30	61	1	2	3	52	20	27	1	22.4.1993	8.8.1991	1	5	4.6	40	1	74.5	52.2	13.4	2
31	52	1	3	3	55	20	33	1	26.5.99	18.9.1991	2	4.3	3.6	58.2	2	81.5	80.8	13.5	2
32	80	1	2	3	52	20	26	1	14.12.1993	27.9.1991	1	5	5.6	32.1	2	51.9	69.8	14.3	3
33	66	1	2	3	52	20	32	1	11.9.1992	5.12.1991	1	3.6	4.6	42	2	42.2	73.6	10.4	2
34	80	1	2	3	52	20	34	1	5.12.1996	28.1.1992	1	4.6	4.3	45.6	2	69.9	54.1	12.2	1
35	68	1	3	3	55	20	25	1	13.6.1994	20.2.1992	1	3	3.3	39.3	1	86.4	70.3	10.5	3
36	76	2	3	3	52	20	38	1	15.3.99	4.3.1992	2	5	6	31.1	2	54.1	66.7	12.2	3
37	72	1	2	3	52	20	32	1	4.12.1994	9.4.1992	1	6.6	5.6	23.8	2	37.6	69.4	11.5	1
38	74	1	2	3	52	20	35	1	9.2.1994	17.6.1992	1	3	3.3	45.5	1	64.8	59.5	14.3	1
39	73	2	3	3	52	20	35	1	16.4.1993	3.12.1992	1	5.3	4.6	44.3	2	72.7	49.2	12.3	2
40	86	1	3	3	50	20	29	1	16.2.1994	26.5.1993	1	4	5	51.9	1	80	78.9	12.8	3
41	85	1	2	2	50	20	27	1	7.3.1996	7.6.1993	1	5.3	5.6	59.7	1	80.1	83.4	11.4	3
42	73	1	2	3	52	20	29	1	30.5.99	7.7.1993	2	3.3	4.6	31.1	1	57.4	52.8	11.8	2
43	59	2	3	3	52	20	33	1	24.4.99	26.8.1994	2	6	3.6	56.5	2	32.7	85.9	12.2	3
44	76	1	3	3	52	20	29	1	8.3.1996	10.9.1994	1	7	5	37.8	1	31.1	55.8	14.8	3
45	75	1	2	3	50.2	20	27	1	3.5.99	19.9.1994	2	3.3	4	41.3	1	70.3	45.6	10.2	1
46	59	1	2	3	55	20	25	1	12.2.99	22.12.1995	2	5.6	7	46.5	1	0	69.2	8.6	1
47	89	2	2	3	50	20	27	2	28.6.96	2.11.94	1	3	3.6	51.7	1	0	76.7	12	2
48	71	1	2	3	54	20	32	2	24.8.98	14.5.92	2	4.3	3.6	42.6	1	45.5	51.9	14.3	2
49	67	1	3	3	54	20	27	2	17.10.98	7.1.93	2	5.3	4.6	32.4	1	85.5	80	15.4	2

50	74	1	2	2	54	20	29	2	25.11.98	25.3.91	2	4.6	5.3	42.7	1	99.8	80	14.4	1
51	68	1	3	3	60	30	48	2	10.5.97	23.5.89	1	5.3	4.3	61.2	2	74.1	67.7	13.7	3
52	75	2	3	3	60	30	52	2	4.8.92	13.11.89	1	5.3	4	47.5	2	41.3	72.9	14.1	3
53	56	2	2	2	52	20	28	2	2.5.99	23.11.89	2	3	2	50.8	2	34.2	63.3	13.5	1
54	34	1	2	3	57.5	23	32	2	8.3.91	3.7.89	2	5.6	4.6	36.3	2	61.8	80.1	11.5	2
55	72	1	3	3	52	20	34	2	23.8.93	2.1.92	1	3.3	3.3	39	2	70.7	56.9	14.2	3
56	68	1	3	3	60	30	41	2	10.4.94	8.2.89	1	5.3	5	30.3	1	0	97.5	10.5	3
57	55	1	2	3	52	20	32	2	3.9.97	18.2.92	1	3.3	5.6	49.5	2	87.4	76.6	13	1
58	79	1	2	3	52	20	36	2	26.1.97	3.12.91	1	3.3	5.6	26.7	1	84.8	67.3	11.6	3
59	77	1	3	3	52	20	35	2	22.5.94	12.3.93	1	4.3	4.6	34.8	2	54.5	68.3	12.3	2
60	75	1	2	3	54	20	28	2	2.5.98	10.2.94	1	4.3	5.6	57.5	2	35.2	74	12.7	1
61	64	1	2	3	54	20	29	2	5.4.96	26.4.94	1	3.6	3.3	26.5	2	90.7	74.8	13.6	2
62	70	1	3	3	54	20	32	2	23.4.94	12.8.93	1	3	2.3	20	1	99.3	84.3	12.7	3
63	52	1	2	3	54	20	28	2	26.7.95	26.7.94	1	3	2.6	54.2	1	0	54.7	14	3
64	74	1	3	3	54	20	32	2	19.2.97	21.12.94	1	4	2.6	25.8	2	43.4	62.2	13.1	3
65	76	1	2	3	60	30	43	2	20.10.98	23.2.95	2	3.3	4	21	1	0	69	13.9	1
66	77	1	2	3	50	20	28	2	3.11.96	8.2.95	1	4.3	3.3	36.1	2	15	55.2	14.3	1
67	78	1	2	3	50	20	29	2	2.6.96	31.3.93	1	3.6	2.6	44.1	1	63.5	65.5	10.1	3
68	69	1	3	3	54	20	29	2	10.8.98	12.8.93	2	2.6	3	31.4	2	68.3	65.3	12.7	3
69	76	1	3	3	50	20	27	2	24.1.94	10.6.93	1	4.6	2.6	44.6	2	99.8	98.6	12.7	1
70	69	1	3	3	50	20	27	2	4.11.94	4.6.92	1	3	2.6	31.2	2	20	41.4	13.9	3
71	60	1	2	2	54	20	29	2	7.11.98	19.8.92	2	5.6	7.6	30	1	96	73.1	12.2	1
72	56	1	2	3	54	20	30	2	3.2.99	14.12.92	2	6.6	5.6	31.9	1	64.8	32.7	13.1	3
73	85	1	2	3	54	20	28	2	11.12.91	8.5.90	1	3.6	4.3	49	2	0	60	11.8	1
74	86	2	3	3	60	30	46	2	21.9.92	20.12.90	1	4.3	3.6	59.1	1	31.1	40	13.3	3

75	79	1	3	3	58	26	37	2	10.5.95	8.11.88	1	4.3	4.6	42.1	2	63.6	50.6	11.8	2
76	36	1	2	3	60	30	43	2	3.4.99	22.2.89	2	5.6	6.6	56.2	2	72.6	34.6	15.3	1
77	72	1	3	3	54	20	31	2	5.1.99	19.12.89	2	5	4.3	55.4	1	0	66.3	14.8	3
78	54	1	3	3	60	30	43	2	26.4.94	25.4.89	1	3.3	3.3	50.8	2	35.5	52.8	13.4	3
79	71	1	3	3	60	30	43	2	7.11.1994	12.7.1989	1	4.3	5.6	56.5	1	85.7	88	9.4	3
80	75	1	3	2	52	20	29	2	7.7.1994	24.10.1989	1	3.3	3.6	42.2	2	0	33.2	12.5	3
81	62	1	3	3	60	30	42	2	30.10.98	31.1.1990	2	4	4.6	43.4	2	56.1	75.2	14.7	3
82	72	1	3	3	52	20	31	2	8.3.1991	2.5.1990	1	5.3	5	35.2	2	59.4	82.4	13.7	2
83	78	1	3	3	52	20	25	2	31.8.1995	15.5.1990	1	4.3	5.3	45.8	1	74.7	93.3	12.5	3
84	66	2	3	3	52	20	27	2	28.12.1993	15.5.1990	1	3.3	4.3	40.5	2	20	60.6	13.1	3
85	67	1	3	3	52	20	30	2	10.2.1995	11.6.1990	1	6.6	5.6	65.4	2	80	65.9	12	2
86	76	1	3	3	52.5	20	30	2	12.5.1991	1.8.1990	1	4	5.3	35.5	1	66.1	69.7	12.4	2
87	72	1	2	3	52	20	29	2	4.2.99	14.2.1992	2	5.6	6	47.6	1	42.2	70.2	14.3	2
88	77	1	2	3	52	20	29	2	2.3.99	24.2.1992	2	5.3	5.3	55.8	2	89.8	64.7	14.1	3
89	64	1	3	3	52	20	35	2	13.2.99	4.9.1992	2	3.6	5.6	51.2	2	0	81	16.7	1
90	64	1	2	3	52	20	25	2	15.3.99	3.6.1993	2	5.6	6.6	49.2	2	70.2	49.2	13.4	2
91	59	1	2	3	50	20	30	2	30.1.99	28.11.1993	2	5	6.6	47.3	2	89.3	88.4	14.5	2
92	69	1	2	3	52	20	27	2	22.3.99	20.1.1994	2	6.3	6.6	52.3	2	67.5	78.6	13	3
93	69	1	2	3	50	20	33	2	12.8.1995	30.3.1994	1	6	7.6	40	2	65.9	75.1	10.5	3
94	68	1	2	3	52	20	27	2	24.3.99	27.4.1994	1	3.6	3.3	30	2	0	78.7	14.1	2
95	76	1	2	3	52	20	29	2	10.2.99	15.9.1994	2	7	8.6	44	1	84.2	77.5	13.6	3
96	81	1	2	2	59	20	27	2	2.2.99	23.9.1994	2	4.3	2.6	57.9	1	87.9	76.6	13.9	3
97	84	1	2	3	52	20	31	2	20.5.1996	14.12.1994	1	3.6	5.6	57.4	1	64	56	10.1	1
98	73	1	3	3	55	20	26	2	16.3.99	3.5.1995	2	4.3	5	38	1	53.7	72.5	13.8	3
99	66	1	2	3	52	20	29	2	22.2.99	5.9.1995	2	5.6	5	56.1	1	42.2	72	13.1	1
100	63	1	3	3	60	30	44	2	3.1.90	27.7.88	1	5	5	48.1	2	34.9	45.6	10.2	3
101	67	1	3	3	54	21	49	2	23.3.99	22.9.1992	2	4	5.3	34.1	2	39.4	55.2	14.7	1

Numb.	Age	Sex	Stage	Grade	Radio.total dos.	Num. Frac.	Days	Cysto.Res.	Last point review	Start.Rad.	Alive	CD 31	CD 34	CD 68	bcl-2	P53	MIB1	Hb	Site
1	62	1	2	2	60	30	49	1	28.12.94	25.4.94	1	4	3.6	49.9	1	60.4	93.8	16	3
2	74	1	2	3	60	30	44	1	23.5.98	5.8.92	2	2.3	5	33.8	2	51.9	81.2	15.2	3
3	71	1	3	3	54	20	32	1	3.7.95	15.12.94	1	3.3	5.3	30.2	1	59.1	44.2	12.5	2
4	79	2	2	3	50	20	27	1	29.11.95	18.1.95	1	4.6	3.6	46.9	1	77.1	96.3	12.8	3
5	53	1	3	3	60	30	42	1	18.12.94	24.5.93	1	4.6	4.3	47	1	43.9	69	11.4	1
6	75	1	3	3	54	20	32	1	10.12.94	7.3.94	1	3	3.3	37.7	1	88.3	51.4	10.1	1
7	55	2	3	3	54	20	30	1	4.4.1995	1.3.94	1	2.6	3.6	40	1	64.5	68.2	13.4	2
8	73	2	3	3	50	25	38	1	22.3.94	28.9.93	1	4	3.3	30.4	1	81.6	96.9	11	2
9	76	1	3	3	50	25	32	1	28.2.94	16.11.92	1	3.3	3.6	32.1	1	21	64.2	11.1	3
10	76	1	3	3	54	20	27	1	30.9.92	3.6.92	1	5.3	4.6	34.9	2	95.9	72.8	12.9	3
11	75	2	3	3	54	20	27	1	30.9.94	8.7.92	1	6.6	6.6	47.7	1	96.2	91	9.8	2
12	50	2	3	3	54	20	32	1	17.9.98	7.5.92	2	5.6	5	25	1	85.4	67.8	14.4	1
13	78	1	3	2	60	30	43	1	15.8.93	9.3.92	1	5.6	5.3	30.2	1	41.5	33.8	11.1	1
14	76	1	2	3	54	20	25	1	20.11.98	24.2.92	2	3.3	3.3	34.9	2	37.7	34.1	15.4	3
15	81	1	2	3	60	30	41	1	1.3.93	13.6.91	1	5.6	6	67.2	1	63.5	54.3	13	2
16	70	1	2	3	54	20	28	1	25.1.94	10.6.91	1	5	5.3	35.6	1	85.5	74.5	12.9	2
17	70	1	3	3	60	30	49	1	28.9.91	19.2.90	1	5	3.6	57.9	2	59	40.6	13.8	2
18	56	2	3	3	58	29	42	1	19.11.93	16.11.89	1	3	3.3	60.8	2	51.9	76.2	13	3
19	74	2	3	3	60	30	44	1	6.10.89	21.3.89	1	4	4.6	36.7	2	59.7	69.9	9.6	3
20	64	1	3	3	60	30	44	1	19.10.97	16.8.89	1	5	5	48.6	2	63.3	79.3	13.8	3
21	74	1	2	3	54	20	29	1	10.2.92	19.9.89	1	6.3	4.6	50.2	2	99.8	82.1	13.2	2
22	55	2	3	3	58.8	29	31	1	19.11.93	16.11.89	1	5.3	4.3	41.8	2	82.5	59.4	13	1
23	64	1	3	3	60	30	45	1	24.11.88	15.8.88	1	3.6	4.3	32.1	2	56.4	71.9	13.2	3
24	74	1	3	2	60	30	45	1	24.1.90	10.3.89	1	3.3	2.6	26.5	2	0	46.2	14.4	1

25	46	1	2	3	60	30	43	1	18.9.91	27.4.89	1	3.6	4.6	39.8	2	98.8	80.4	13.4	2
26	73	1	2	3	52	20	28	1	12.4.1992	3.1.1990	1	3	5	41	1	52.8	70.6	10.9	3
27	69	1	2	2	52.5	20	25	1	3.5.99	24.1.1990	2	4	3.3	50	1	80.8	90	12.7	2
28	70	1	3	3	50	20	38	1	15.11.1990	28.3.1990	1	4.3	5.6	26	2	67.5	60.1	11.3	2
29	65	1	3	2	55	20	27	1	14.4.99	5.6.1990	2	3	4.3	37.6	1	70	50.4	9.3	3
30	61	1	2	3	52	20	27	1	22.4.1993	8.8.1991	1	5	4.6	40	1	74.5	52.2	13.4	2
31	52	1	3	3	55	20	33	1	26.5.99	18.9.1991	2	4.3	3.6	58.2	2	81.5	80.8	13.5	2
32	80	1	2	3	52	20	26	1	14.12.1993	27.9.1991	1	5	5.6	32.1	2	51.9	69.8	14.3	3
33	66	1	2	3	52	20	32	1	11.9.1992	5.12.1991	1	3.6	4.6	42	2	42.2	73.6	10.4	2
34	80	1	2	3	52	20	34	1	5.12.1996	28.1.1992	1	4.6	4.3	45.6	2	69.9	54.1	12.2	1
35	68	1	3	3	55	20	25	1	13.6.1994	20.2.1992	1	3	3.3	39.3	1	86.4	70.3	10.5	3
36	76	2	3	3	52	20	38	1	15.3.99	4.3.1992	2	5	6	31.1	2	54.1	66.7	12.2	3
37	72	1	2	3	52	20	32	1	4.12.1994	9.4.1992	1	6.6	5.6	23.8	2	37.6	69.4	11.5	1
38	74	1	2	3	52	20	35	1	9.2.1994	17.6.1992	1	3	3.3	45.5	1	64.8	59.5	14.3	1
39	73	2	3	3	52	20	35	1	16.4.1993	3.12.1992	1	5.3	4.6	44.3	2	72.7	49.2	12.3	2
40	86	1	3	3	50	20	29	1	16.2.1994	26.5.1993	1	4	5	51.9	1	80	78.9	12.8	3
41	85	1	2	2	50	20	27	1	7.3.1996	7.6.1993	1	5.3	5.6	59.7	1	80.1	83.4	11.4	3
42	73	1	2	3	52	20	29	1	30.5.99	7.7.1993	2	3.3	4.6	31.1	1	57.4	52.8	11.8	2
43	59	2	3	3	52	20	33	1	24.4.99	26.8.1994	2	6	3.6	56.5	2	32.7	85.9	12.2	3
44	76	1	3	3	52	20	29	1	8.3.1996	10.9.1994	1	7	5	37.8	1	31.1	55.8	14.8	3
45	75	1	2	3	50.2	20	27	1	3.5.99	19.9.1994	2	3.3	4	41.3	1	70.3	45.6	10.2	1
46	59	1	2	3	55	20	25	1	12.2.99	22.12.1995	2	5.6	7	46.5	1	0	69.2	8.6	1
47	89	2	2	3	50	20	27	3	28.6.96	2.11.94	1	3	3.6	51.7	1	0	76.7	12	2
48	71	1	2	3	54	20	32	3	24.8.98	14.5.92	2	4.3	3.6	42.6	1	45.5	51.9	14.3	2
49	67	1	3	3	54	20	27	3	17.10.98	7.1.93	2	5.3	4.6	32.4	1	85.5	80	15.4	2

50	74	1	2	2	54	20	29	3	25.11.98	25.3.91	2	4.6	5.3	42.7	1	99.8	80	14.4	1
51	68	1	3	3	60	30	48	3	10.5.97	23.5.89	1	5.3	4.3	61.2	2	74.1	67.7	13.7	3
52	75	2	3	3	60	30	52	3	4.8.92	13.11.89	1	5.3	4	47.5	2	41.3	72.9	14.1	3
53	56	2	2	2	52	20	28	3	2.5.99	23.11.89	2	3	2	50.8	2	34.2	63.3	13.5	1
54	34	1	2	3	57.5	23	32	3	8.3.91	3.7.89	2	5.6	4.6	36.3	2	61.8	80.1	11.5	2
55	72	1	3	3	52	20	34	3	23.8.93	2.1.92	1	3.3	3.3	39	2	70.7	56.9	14.2	3
56	68	1	3	3	60	30	41	3	10.4.94	8.2.89	1	5.3	5	30.3	1	0	97.5	10.5	3
57	55	1	2	3	52	20	32	3	3.9.97	18.2.92	1	3.3	5.6	49.5	2	87.4	76.6	13	1
58	79	1	2	3	52	20	36	3	26.1.97	3.12.91	1	3.3	5.6	26.7	1	84.8	67.3	11.6	3
59	77	1	3	3	52	20	35	3	22.5.94	12.3.93	1	4.3	4.6	34.8	2	54.5	68.3	12.3	2
60	75	1	2	3	54	20	28	2	2.5.98	10.2.94	1	4.3	5.6	57.5	2	35.2	74	12.7	1
61	64	1	2	3	54	20	29	2	5.4.96	26.4.94	1	3.6	3.3	26.5	2	90.7	74.8	13.6	2
62	70	1	3	3	54	20	32	2	23.4.94	12.8.93	1	3	2.3	20	1	99.3	84.3	12.7	3
63	52	1	2	3	54	20	28	2	26.7.95	26.7.94	1	3	2.6	54.2	1	0	54.7	14	3
64	74	1	3	3	54	20	32	2	19.2.97	21.12.94	1	4	2.6	25.8	2	43.4	62.2	13.1	3
65	76	1	2	3	60	30	43	2	20.10.98	23.2.95	2	3.3	4	21	1	0	69	13.9	1
66	77	1	2	3	50	20	28	2	3.11.96	8.2.95	1	4.3	3.3	36.1	2	15	55.2	14.3	1
67	78	1	2	3	50	20	29	2	2.6.96	31.3.93	1	3.6	2.6	44.1	1	63.5	65.5	10.1	3
68	69	1	3	3	54	20	29	2	10.8.98	12.8.93	2	2.6	3	31.4	2	68.3	65.3	12.7	3
69	76	1	3	3	50	20	27	2	24.1.94	10.6.93	1	4.6	2.6	44.6	2	99.8	98.6	12.7	1
70	69	1	3	3	50	20	27	2	4.11.94	4.6.92	1	3	2.6	31.2	2	20	41.4	13.9	3
71	60	1	2	2	54	20	29	2	7.11.98	19.8.92	2	5.6	7.6	30	1	96	73.1	12.2	1
72	56	1	2	3	54	20	30	2	3.2.99	14.12.92	2	6.6	5.6	31.9	1	64.8	32.7	13.1	3
73	85	1	2	3	54	20	28	2	11.12.91	8.5.90	1	3.6	4.3	49	2	0	60	11.8	1
74	86	2	3	3	60	30	46	2	21.9.92	20.12.90	1	4.3	3.6	59.1	1	31.1	40	13.3	3

75	79	1	3	3	58	26	37	2	10.5.95	8.11.88	1	4.3	4.6	42.1	2	63.6	50.6	11.8	2
76	36	1	2	3	60	30	43	2	3.4.99	22.2.89	2	5.6	6.6	56.2	2	72.6	34.6	15.3	1
77	72	1	3	3	54	20	31	2	5.1.99	19.12.89	2	5	4.3	55.4	1	0	66.3	14.8	3
78	54	1	3	3	60	30	43	2	26.4.94	25.4.89	1	3.3	3.3	50.8	2	35.5	52.8	13.4	3
79	71	1	3	3	60	30	43	2	7.11.1994	12.7.1989	1	4.3	5.6	56.5	1	85.7	88	9.4	3
80	75	1	3	2	52	20	29	2	7.7.1994	24.10.1989	1	3.3	3.6	42.2	2	0	33.2	12.5	3
81	62	1	3	3	60	30	42	2	30.10.98	31.1.1990	2	4	4.6	43.4	2	56.1	75.2	14.7	3
82	72	1	3	3	52	20	31	2	8.3.1991	2.5.1990	1	5.3	5	35.2	2	59.4	82.4	13.7	2
83	78	1	3	3	52	20	25	2	31.8.1995	15.5.1990	1	4.3	5.3	45.8	1	74.7	93.3	12.5	3
84	66	2	3	3	52	20	27	2	28.12.1993	15.5.1990	1	3.3	4.3	40.5	2	20	60.6	13.1	3
85	67	1	3	3	52	20	30	2	10.2.1995	11.6.1990	1	6.6	5.6	65.4	2	80	65.9	12	2
86	76	1	3	3	52.5	20	30	2	12.5.1991	1.8.1990	1	4	5.3	35.5	1	66.1	69.7	12.4	2
87	72	1	2	3	52	20	29	2	4.2.99	14.2.1992	2	5.6	6	47.6	1	42.2	70.2	14.3	2
88	77	1	2	3	52	20	29	2	2.3.99	24.2.1992	2	5.3	5.3	55.8	2	89.8	64.7	14.1	3
89	64	1	3	3	52	20	35	2	13.2.99	4.9.1992	2	3.6	5.6	51.2	2	0	81	16.7	1
90	64	1	2	3	52	20	25	2	15.3.99	3.6.1993	2	5.6	6.6	49.2	2	70.2	49.2	13.4	2
91	59	1	2	3	50	20	30	2	30.1.99	28.11.1993	2	5	6.6	47.3	2	89.3	88.4	14.5	2
92	69	1	2	3	52	20	27	2	22.3.99	20.1.1994	2	6.3	6.6	52.3	2	67.5	78.6	13	3
93	69	1	2	3	50	20	33	2	12.8.1995	30.3.1994	1	6	7.6	40	2	65.9	75.1	10.5	3
94	68	1	2	3	52	20	27	2	24.3.99	27.4.1994	1	3.6	3.3	30	2	0	78.7	14.1	2
95	76	1	2	3	52	20	29	2	10.2.99	15.9.1994	2	7	8.6	44	1	84.2	77.5	13.6	3
96	81	1	2	2	59	20	27	2	2.2.99	23.9.1994	2	4.3	2.6	57.9	1	87.9	76.6	13.9	3
97	84	1	2	3	52	20	31	2	20.5.1996	14.12.1994	1	3.6	5.6	57.4	1	64	56	10.1	1
98	73	1	3	3	55	20	26	2	16.3.99	3.5.1995	2	4.3	5	38	1	53.7	72.5	13.8	3
99	66	1	2	3	52	20	29	2	22.2.99	5.9.1995	2	5.6	5	56.1	1	42.2	72	13.1	1
100	63	1	3	3	60	30	44	2	3.1.90	27.7.88	1	5	5	48.1	2	34.9	45.6	10.2	3
101	67	1	3	3	54	21	49	2	23.3.99	22.9.1992	2	4	5.3	34.1	2	39.4	55.2	14.7	1

LITERATURE CITED

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